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13. ABSTRACT *(Maximum 200 words)*

The goal of this research was to develop analytical methods capable of determining the concentration of toxic (bioavailable) forms of copper in natural surface waters. The approach should also be applicable to other metals. The approach was: (1) to determine the apparent binding affinity of the gills of fish and other aquatic biota for copper using novel competition bioassay and copper residue accumulation techniques; and (2) to modify the performance of commercial cation exchange resins or synthesize custom-made cation exchange resins, to match the copper binding affinity of fish and other aquatic biota. Using a range of procedures, the apparent copper binding affinities (log of the Apparent Binding Affinity (ABA)) were determined for rainbow trout gills (6.4-7.2), brook trout gills (7.1-7.2), trout mucus (6.9-7.7), and *Daphnia magna* (6.6-8.1). Based on these results an acceptable value for log ABA would be 7.6 for cation-exchange chromatography procedures to measure the bioavailable fraction of copper. Commercially available resins under a variety of conditions consistently had copper binding affinities that were 2 to 3 orders of magnitude higher than the measured values for aquatic biota. Custom cation exchange resins were synthesized and yielded binding affinities closer to that of aquatic biota, but additional work is needed to standardize and validate this approach.

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The overall goal of this research project has been to develop an analytical method capable of determining the concentration of toxic (bioavailable) forms of metals in natural surface waters. Such a method is needed because existing methods recommended for use by the U.S. Environmental Protection Agency (the "total recoverable method") can over-estimate the toxic, bioavailable fraction of metals, resulting in costly over-protection in regulated surface waters of the U.S. The theoretical and procedural bases of the research project were modified from earlier work in this laboratory with aluminum (Kline 1992, Fernandez 1994). Research in the current AFOSR project has emphasized copper with possible applications to other metals.

The overall approach was: (1) to determine the binding affinity of the gills of fish and other aquatic animals for copper using a novel competition bioassay technique and traditional copper residue accumulation techniques; and (2) to develop cation exchange chromatography techniques to match the copper binding affinity (and bioavailability) for aquatic biota.

(a) Objectives of Research

The specific research objectives and tasks of this AFOSR project, as expanded and reorganized from those presented in the proposal, were as follows:

(1) Compile a library of copper-organic acid stability constants from the published geochemical literature for a series of organic acids that could be used in competition bioassays with copper, organic acids and aquatic biota.

(2) Establish experimental conditions to be used in competition bioassays with copper, organic acids and aquatic biota.

(3) Determine the copper binding affinity for fish and other aquatic biota using competition bioassays and tissue copper residue accumulation techniques.

(4) Modify cation exchange chromatography procedures to match the copper binding affinity for aquatic biota.

(b) Status of Research Effort

(1) Copper-Organic Acid Stability Constants

To establish appropriate water quality conditions for the competition bioassays and to select organic acid ligands with a range of copper binding affinities, copper speciation was calculated for a number of water quality conditions and a series of organic acid ligands. The calculations were performed on a Microsoft Excel spreadsheet. The basic approach was to compile a list of all metal species of interest

and to establish their relationships using stability constants from the literature (e.g., Sillen and Martell 1964, Smith and Martell 1974-1982). Mass balance restrictions then yielded a nonlinear system of equations which were solved in an iterative calculation cycle.

This approach allowed calculations of the interactions between two metals and two ligands. A typical calculation could involve copper, calcium (relevant because it is present at much higher concentrations than copper in the competition bioassays), the major organic acid ligand, and another ligand (chloride, sulfate, etc.) potentially interfering with the copper complexes of interest. Also included were metal-hydroxy complexes, carbonate complexes, and an activity correction (extended Debye-Huckel).

Examples of the results of these calculations for four different organic acid ligands (with two acids at two different acid/copper ratios) are summarized in Table 1 for pH 6 and in Figures 1, 2 and 3 for a pH range of 5 to 7. It is evident from these tabulations and plots that only relatively strong ligands (e.g., as strong as or stronger than citric acid with a copper-organic acid stability constant of 7.17) will bind copper strongly enough to substantially reduce free copper concentrations. It is also evident from Figures 1, 2 and 3 that the binding behavior is pH-dependent and the optimum pH for binding copper is slightly different for each organic acid, but they are all near optimum around pH 6. Also note that calcium concentrations are about 1000 times higher than total copper concentrations in these calculations and in the competition bioassays conducted during this project. The relative amount of calcium bound to the organic ligand is higher for ligands that only contain oxygen functional groups (e.g., citric acid), as opposed to ligands where there are also nitrogen groups available (e.g., NTA, 2,6-pyridinedicarboxylic acid).

(2) Experimental Conditions for Competition Bioassays

Prior to determining the copper binding affinity of fish gills using copper-fish-organic acid competition bioassays, it was first necessary to optimize experimental conditions, particularly with respect to copper and calcium concentrations to be used in the bioassays. Appropriately toxic copper concentrations were selected so that organic acids with different copper binding affinities (strong affinity and protective to fish versus weak affinity and not protective) could be compared on the basis of fish mortality. Also, since calcium can compete with copper for binding sites on fish gills as well as for binding sites on organic acids, it was necessary to select an optimal calcium concentration so that calculation of metal complexation and speciation could be as straightforward as possible.

(2.1) Copper incipient lethal level (ILL)

Three sets of experiments were performed to determine the ILL (incipient lethal level or time-independent LC_{50}) of copper for rainbow trout (*Oncorynchus mykiss*). The current USEPA water quality criteria document for copper (USEPA 1985) and available published literature on copper toxicity all report LC_{50} values for fish based on

Table 1. Complex formation between copper and four organic acid ligands with copper at 5 ppb, calcium at 5 ppm, pH 6 and with organic acid:copper ratios of 5:1 and 1:1. See Figures 1, 2 and 3 for plotted concentrations of inorganic copper and copper complexes at pH values from 5 through 7.

Complex Formation between Copper and Selected Ligands

total Cu 5 ppb
total Ligand in excess of copper, as indicated

total Ca 5 ppm
other solutes: NaCl, ~2 ppm

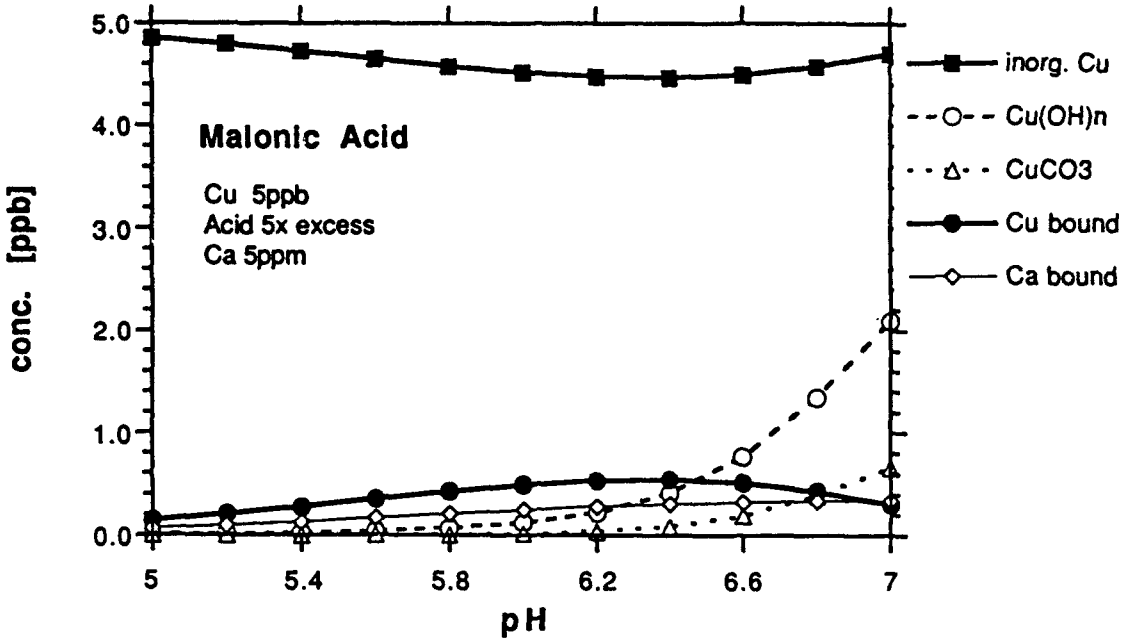
pH 6

	Malonic Acid	Citric Acid	2,6-Pyridine-dicarbox. A.	2,6-Pyridine-dicarbox. A.	NTA	NTA
excess acid:	5x	5x	1x	5x	1x	5x
log K	5.7	7.17	9.94	9.94	14.37	14.37
highest pK	5.696	6.396	5.07	5.07	10.334	10.334
Inorg. Cu 1)	**4.51**	**3.33**	**1.07**	**0.05**	**0.20**	**0.00**
Cu(OH)n 2)	0.12	0.09	0.03	0.00	0.01	0.00
CuCO3	0.02	0.01	0.00	0.00	0.00	0.00
Cu bound 3)	**0.49**	**1.67**	**3.93**	**4.95**	**4.80**	**5.00**
Ca bound 3)	0.25	8.82	0.59	11.16	0.02	2.17
free Lig.	39.50	28.28	0.09	1.77	0.50	49.83
	all concentrations as ppb					

Expressed as % of total Cu, Ca, or Acid

Inorg. Cu 1)	90.3 %	66.6 %	21.3 %	1.0 %	4.0 %	0.0 %
Cu(OH)n 2)	2.4 %	1.8 %	0.6 %	0.0 %	0.1 %	0.0 %
CuCO3	0.3 %	0.2 %	0.1 %	0.0 %	0.0 %	0.0 %
Cu bound 3)	**9.7 %**	**33.4 %**	**78.7 %**	**99.0 %**	**96.0 %**	**100.0 %**
Ca bound 3)	0.0 %	0.2 %	0.0 %	0.2 %	0.0 %	0.0 %
free Lig.	96.5 %	37.4 %	0.7 %	2.7 %	3.3 %	66.3 %

1) sum of Cu2+, all hydroxy species, and carbonate complex
2) sum of hydroxy species
3) sum of Cu/Ca bound to acid



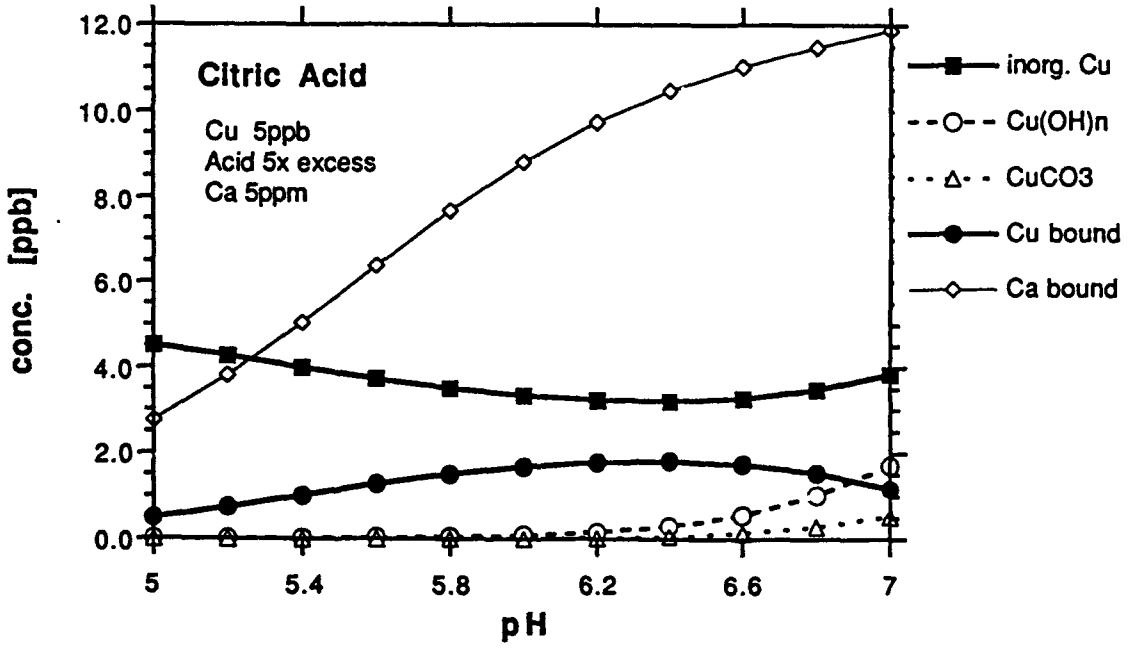


Figure 1. Copper complexation by malonic and citric acids with organic acid:copper ratios of 5:1 and at pH 5 through 7.

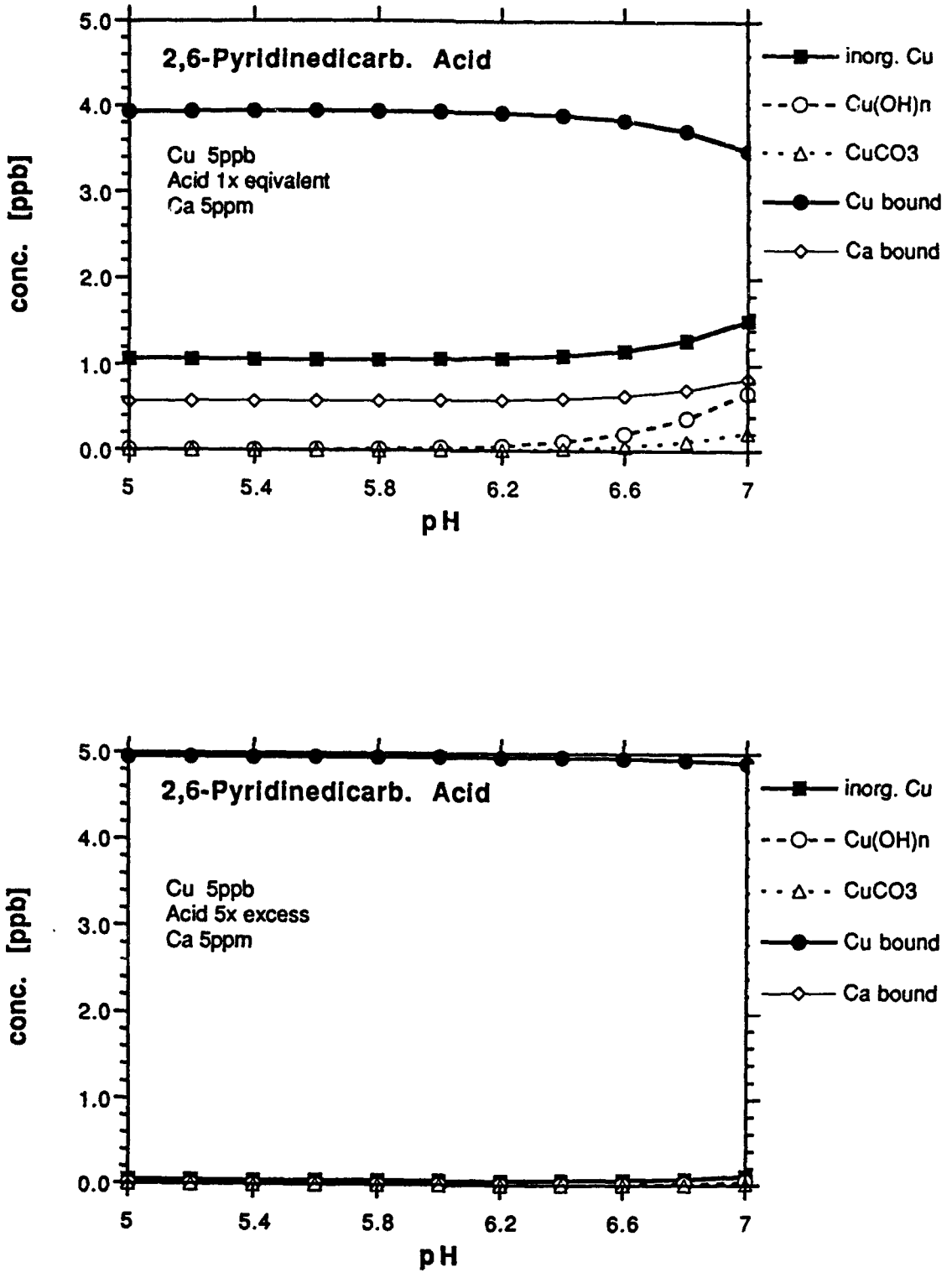


Figure 2. Copper complexation by 2,6-pyridinedicarboxylic acid with organic acid:copper ratios of 1:1 (upper panel) and 5:1 (lower panel) and at pH 5 through 7.

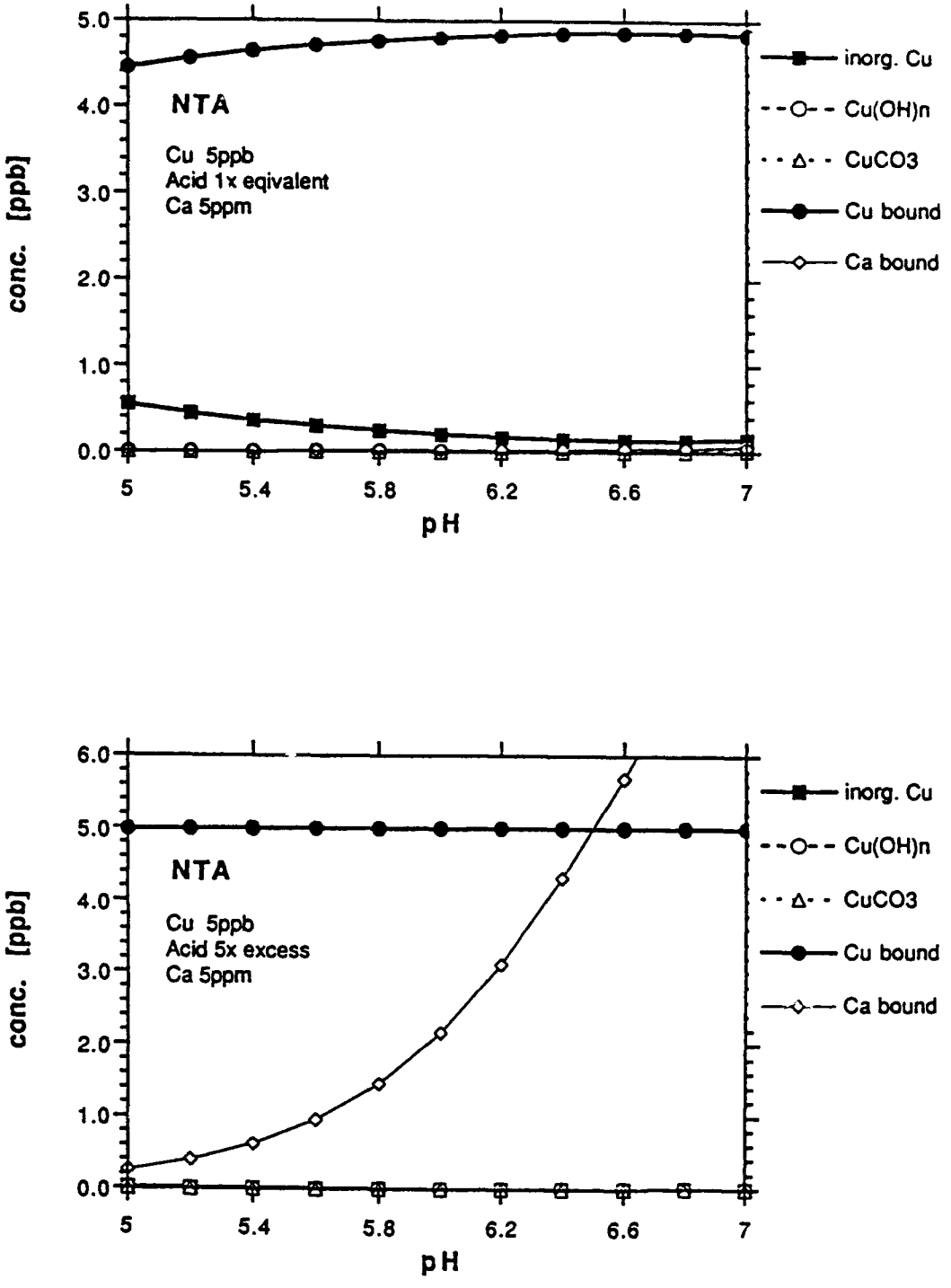


Figure 3. Copper complexation by NTA with organic acid:copper ratios of 1:1 (upper panel) and 5:1 (lower panel) and at pH 5 through 7.

relatively high calcium concentrations (e.g., water hardness values of 30 mg/l as $CaCO_3$, or higher). However, for the competition bioassays under objective 3, above, to be most successful, it was favorable to expose fish to mixtures of copper and organic acids at very low calcium concentrations so that calcium would not outcompete copper for the organic acid ligand. Therefore, the ILL for copper was determined at a calcium concentration of 1 mg/l. From the results shown in Figure 4, the ILL under these conditions was calculated to be 6.9 μg/l copper, and the time to the ILL was 20.6 hours.

(2.2) Calcium acclimation effects on copper toxicity

Two sets of experiments were performed to determine the effect of small differences in calcium concentration and the effect of different levels of calcium acclimation on copper toxicity. As mentioned earlier, the ameliorating effects of calcium on heavy metal toxicity have been well documented. In spite of this, there is very little data available to estimate the influence of small differences in calcium concentration (particularly at very low calcium concentrations in the range of 1 to 10 mg/l) on copper toxicity. Moreover, even less data are available to determine the effect of acclimation to different calcium concentrations on copper toxicity. Thus, to select the optimum calcium concentration for fish acclimation and for conducting the competition bioassays, it was necessary to conduct the two sets of experiments described below.

In the calcium exposure experiment, rainbow trout were acclimated for five months to 1 mg/l calcium and then placed in 3 μg/l copper at each of five different calcium concentrations (1, 2.5, 3.5, 6.5, and 10.5 mg/l calcium). The results plotted as percent survival versus time (Figure 5, upper panel) clearly show a marked protective effect of calcium on copper toxicity. This protective effect of calcium is also demonstrated with the results presented as percent survival at 96 hours and as the LT_{50} (the time to 50 percent mortality), as shown in Figure 6 (cross-hatched bars in both upper and lower panels). It is also clear from Figure 5 (upper panel) and Figure 6 (cross-hatched bars) that increased calcium concentration from 1 though 3.5 mg/l produced stepwise increases in survival, and that 6.5 and 10.5 mg/l calcium completely protected rainbow trout from the lethal effects of 3 μg/l copper under these experimental conditions.

In the experiment to determine effects of different calcium acclimation concentrations on copper toxicity, rainbow trout were acclimated to treatment calcium concentrations (1, 2.5, 3.5, 6.5, and 10.5 mg/l calcium) for fourteen days prior to the same exposure regimen used in the previous experiment (3 μg/l copper at each of the 5 calcium concentrations). The results, this time, were markedly different than when fish were all acclimated to only 1 mg/l calcium. As is evident from Figure 5 (lower panel) and Figure 6 (solid bars), there was no consistent difference in fish mortality in the different calcium acclimation/exposure treatment concentrations. And, remarkably, the fish from higher calcium acclimation concentrations (> 1 mg/l calcium) were <u>more</u> sensitive to copper toxicity than were fish acclimated to only 1 mg/l

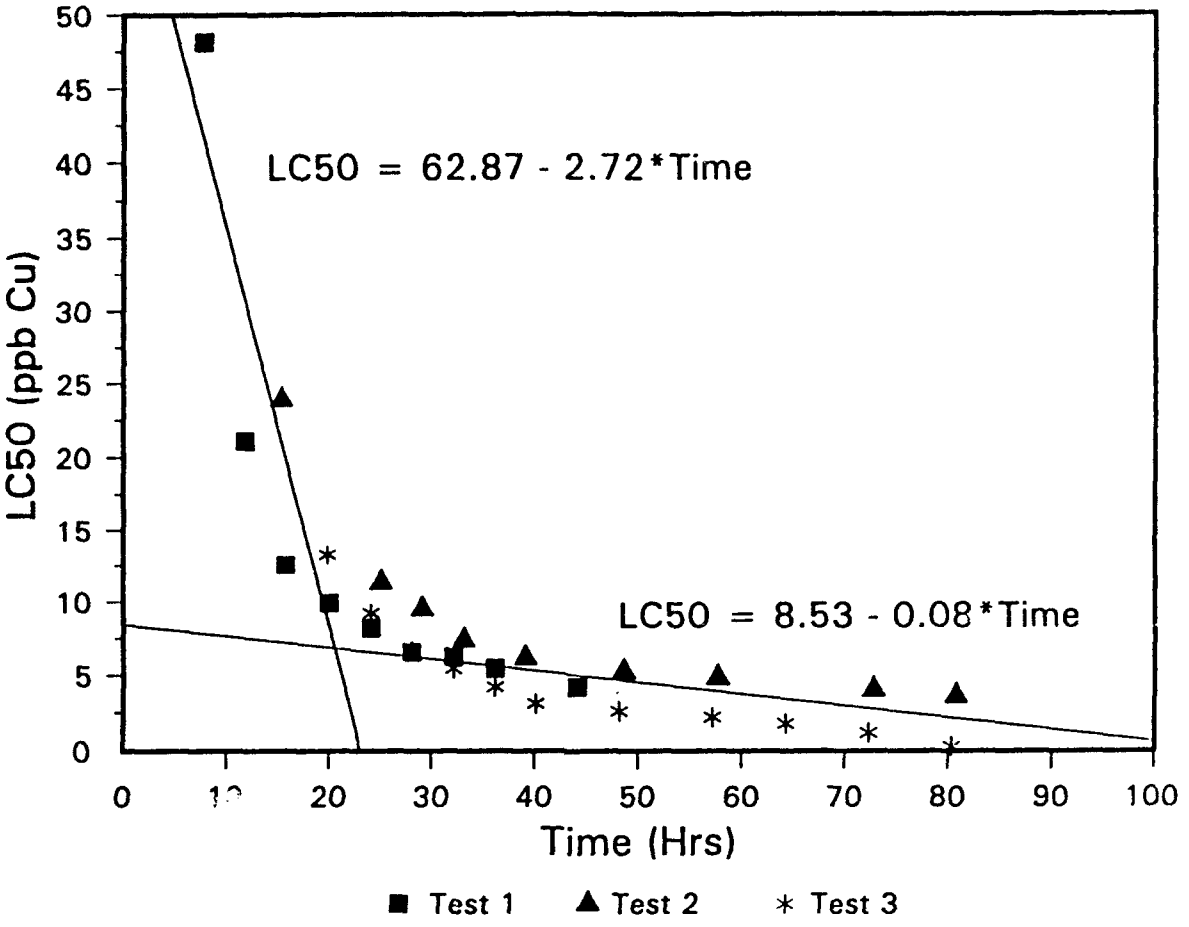


Figure 4. Determination of the copper Incipient Lethal Level (ILL) with data combined from three experiments conducted at 1 ppm calcium and pH 6. ILL estimate equals 6.9 ppb copper at 20.6 hours as determined by the intersection of linear regressions on the two curve components.

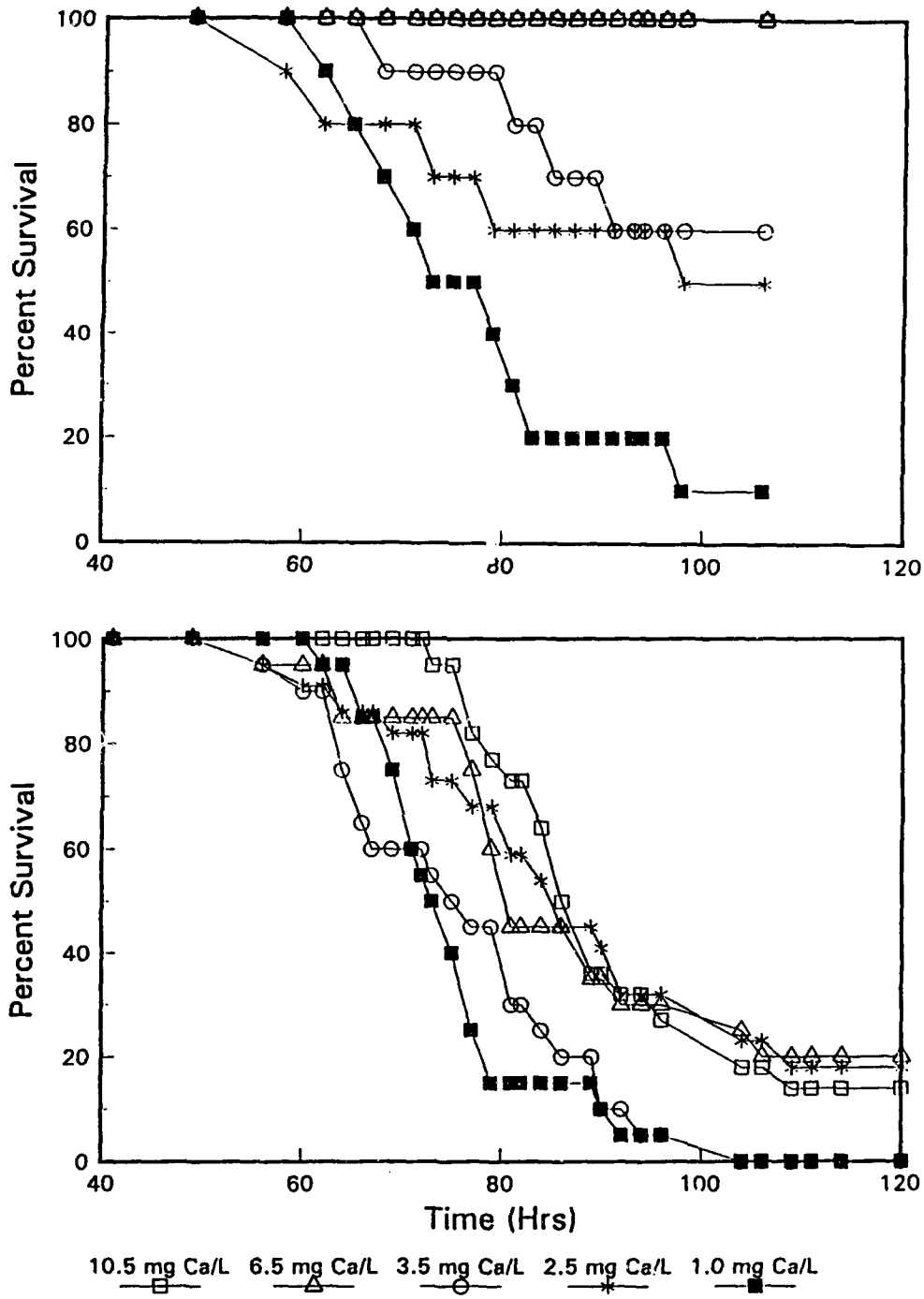


Figure 5. Survival plots for rainbow trout exposed to 3 ppb copper and a series of calcium concentrations from 1.0 to 10.5 ppm. The top panel illustrates results with fish acclimated to 1.0 ppm calcium for 5 months; the bottom panel illustrates results with fish acclimated to exposure calcium concentrations (i.e., 1.0, 2.5, 3.5, 6.5 or 10.5 ppm) for 14 days prior to copper/calcium exposure.

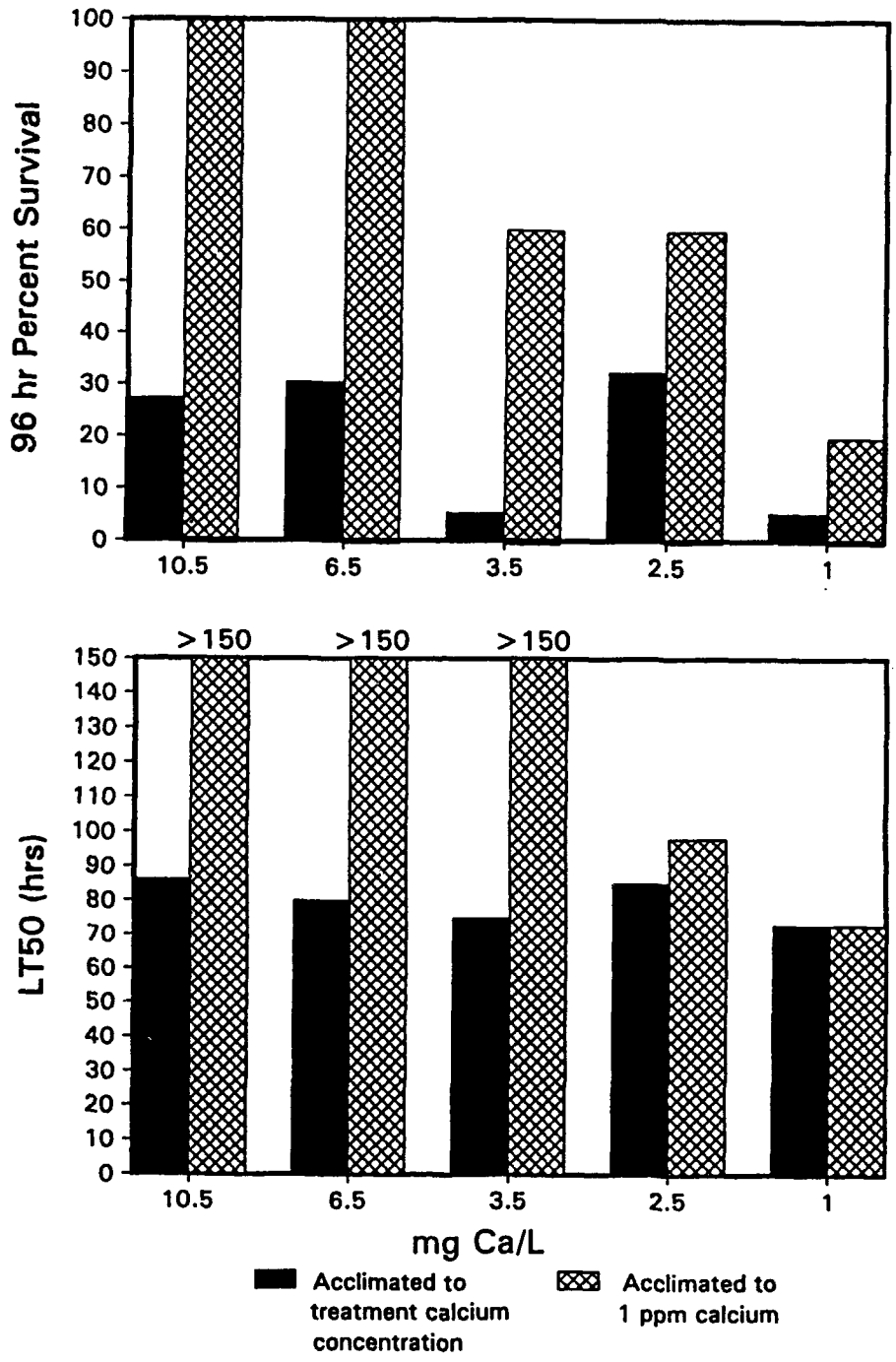


Figure 6. Survival/mortality responses of rainbow trout exposed to 3 ppb copper at a series of calcium concentrations, with fish either acclimated to exposure treatment calcium levels for 14 days (solid bars) or acclimated to 1 ppm calcium (cross-hatched bars) prior to copper exposure. The top panel illustrates percent survival at 96 hours; the bottom panel illustrates time to 50 percent mortality (LT50).

calcium, when both groups were tested at identical calcium concentrations.

There are a number of possible explanations for these results. For instance, we know from previous research in our laboratory and from the published literature that very low calcium concentrations (0.5 to 1 mg/l) stimulate a number of gill structural changes including an increase in the number and total volume density of mucous cells and, presumably, this could lead to an increase in mucus secretion when the fish are subsequently exposed to copper. Whatever the explanation for these results, however, the practical consequences of the results, as they apply to the copper-fish-organic acid competition bioassays, are straightforward. From these experiments we concluded that the competition bioassays should be conducted as follows: (1) calcium concentrations for the acclimation and exposure periods should be identical to simplify interpretation of fish mortality responses to copper exposure; and (2) the calcium concentration selected for these experiments should be 3 to 5 mg/l for a copper exposure concentration of 3 to 10 μg/l.

(3) Copper Binding Affinity for Fish and Other Aquatic Biota

(3.1) Introduction

By using a novel "competition bioassay" approach to determine the effect of organic acid compexation on copper toxicity, combined with the more traditional Scatchard and non-linear regression (NLR) analyses (Hill 1910, Scatchard 1949, Titeler 1981, Norusis 1993) of gill copper accumulation, we sought to define toxicologically relevant apparent binding affinities (conditional stability constants) of copper for rainbow and brook trout gills. We also used Scatchard/NLR analyses to evaluate the copper binding affinity of rainbow trout mucus and the invertebrate, *Daphnia magna*.

The *Daphnia magna* experiments addressed our broader hypothesis, that many aquatic organisms have similar membrane binding characteristics to that of fish, because the gills or external membranes of numerous aquatic organisms such as daphnids, algae, and bacteria may have surface metal-binding constituents in common. Consequently, this would imply that a common definition of bioavailable copper would be possible to encompass a wide diversity of aquatic organisms, and a single cation-exchange resin may then have broad applicability to assessing bioavailable metal for many key indicator species.

The Scatchard/NLR approach also allowed comparison of this study's competition-bioassay determined gill-copper binding affinity to the gill-copper binding affinities determined by two related studies employing Scatchard-based experimental approaches (Reid and McDonald 1991, Playle and Dixon 1994a). Both Scatchard and NLR analyses rely on direct measurement of copper accumulation (presumably Cu^{+2}) on the gills and/or gill mucus coating. However, none of these published studies have verified that the methods yield gill-copper and/or gill-mucus-copper binding constants that are toxicologically meaningful. In contrast, our "competition bioassay"

experimental design establishes a gill-copper binding affinity directly from the outcome of toxicity evaluations.

(3.2) Competition bioassays

(3.2.1) Experimental approach and methods

Gill ligands are composed primarily of phospholipids with amino, phospho, and carboxylate functional groups (Seimiya and Ohki 1973, Bolis et al. 1984) and an associated external mucus layer composed of polyanionic mucopolysaccharides and glycoproteins (Wold and Selset 1977, Van de Winkel et al. 1986). Each have characteristic metal and proton binding constants and capacities that collectively define the copper binding affinity of the gill (Miller and Mackay 1982, Part and Lock 1983, Reid and McDonald 1991). The competition bioassay approach used in this study was derived from observations that organic ligands such as nitrilotriacetic acid (NTA) (Shaw and Brown 1974) and EDTA (Tabata and Nishikawa 1969) can protect fish from the toxic effects of copper by chelating the copper and rendering it non-bioavailable. The gill does not bind Cu-organic complexes such as Cu-EDTA, Cu-NTA, and Cu-Citrate (Playle and Dixon 1994b). Therefore, the apparent copper binding affinity of these aqueous organic acids must be greater than the gill ligands. Conversely, if the gill-ligand affinity for copper were sufficiently greater than that of an external competing ligand, copper should re-speciate from the competing ligand onto the gill, resulting in mortality. Consequently, a fish gill-copper binding affinity could be determined by comparison to the lowest binding affinity organic acid that competes effectively with the fish gill for copper, and thus, prevents mortality.

Juvenile rainbow trout (*Oncorhynchus mykiss*) (15-40g) were acquired from the Dan Speas Hatchery, Casper, WY, and Cline's Hatchery, Boulder, CO. All fish were reared in hard water, held in relatively hard well water (approximately 250 mg/l $CaCO_3$ hardness, 52 mg Ca/l, pH 7.9), then acclimated for 4-24 weeks to the same artificial soft water used during previous exposures described above in Section b.2. Previous studies at this laboratory indicate modifications of the gill binding environment, such as increased chloride cell density and surface area (Laurent and Hebibi 1989, Laurent and Perry 1991, Perry et al. 1992), are complete within a 4-week minimum acclimation time. Fish were fed trout chow daily (Nelson's Silver Cup No.4 coarse) during holding and acclimation, but were not fed 1-2 days before and during experiments. Food was withheld to reduce non-specific copper binding to excreted organic matter.

Before beginning the competition bioassays, a copper concentration was determined that resulted in mortality greater than 75% after 5 days without any competing organics (see Section b.2, above). This copper concentration was then used during all subsequent competition bioassays and also served as a positive control. Therefore, we expected survival similar to this positive control if an added organic acid had a copper binding affinity weaker than that of the fish gill, and we

expected survival similar to a clean-water control if the organic acid had a copper binding affinity greater than that of the fish gill.

Next, in a series of five-day toxicity tests, fish were exposed to this lethal copper concentration in the presence of one of several well-defined organic acids of known copper binding affinity (Table 3). The concentrations of the various organic acids used were sufficient to reduce free Cu^{+2} ion below the five-day lethal threshold (i.e., only 0.1-1 μg/l of the 10 μg/l added copper would remain uncomplexed by the organic acids). Therefore, less than 90% survival would indicate that the fish gill is removing copper from the competing organic. By exposing different groups of fish to copper in the presence of each of a series of organic acids of different copper binding affinity, an overall gill binding affinity could be assigned by comparison to the binding affinity of the organic acid that reduced gill copper accumulation (see below) and maintained survival near 100 percent. The logarithm of the *apparent* copper binding affinity (the effective binding constant, accounting for the nominal exposure water chemistry) ranged from 10.3 (NTA) to 4.2 (Tartaric Acid) (Table 2). The organic acid concentrations required were determined using the geochemical speciation program MINTEQA2, Version 3.11 (Allison et al. 1991). To maintain any unbound copper in the Cu^{+2} form, pH was maintained at 6.5 and background water chemistry was kept as simple as possible to reduce both the influence of inorganic species on copper-organic ligand complexation and any potential competitive interactions between copper and components such as Ca^{+2} or H^{+} for the gill surface. Measured ion concentrations were used to refine initial speciation calculations and apparent binding affinities, and were used in all data analyses and presentations. Details of the water chemistry during the acclimation and exposure periods are presented in Tables 3 and 4.

Two independent competition bioassay experiments were conducted with rainbow trout (RBT): Experiment 1 with fish from Dan Speas Hatchery and Experiment 2 with fish from Cline's Hatchery. A typical bioassay set within each experiment consisted of 25 (Experiment 1) or 30 (Experiment 2) fish in each of six different exposure tanks: 2 copper-organic mixtures (each organic having a different copper binding affinity), 2 organic only controls, 1 copper only control, and 1 clean water control. Mortality (no opercular movement) was recorded at eight-hour intervals, and dead fish were immediately removed, weighed, and measured.

In addition to measuring mortality during these competition bioassays, gills were sampled from surviving fish after 24 hours exposure to determine copper accumulation on the gills. This was done to allow correlations between gill copper accumulation at 24 hours and mortality at 5 days. For these gill samples, fish were sacrificed by pithing, and gill baskets were removed and briefly rinsed in deionized water. Gill filaments were dissected away from the cartilaginous gill arches and digested overnight in 70% nitric acid, diluted to 25 ml with 18 MOhm deionized water and analyzed for copper content.

Table 2. Complex Formation Between Copper and Selected Ligands. Calculations are based on nominal water chemistry. Total Cu 10 ug/l; pH 6.50; Ca 5 mg/l; Na 2 mg/l. Apparent Binding Constant (Log K) = [CuLigand]/ [Cu]*[Ligand], where [Cu] = Total Cu-[CuLigand] and [Ligand] = [Ligand total]-[CuLigand]

Organic acid	Functional group	Apparent Log K_{Cu}	Free Cu^{+2} (μg/l)
Nitrilotriacetic (NTA)	Aliphatic (N center)/3 COO-	10.3	0.10
2,6-Pyridine-dicarboxcylic	Aromatic (N @ C2)/2 COO-	8.6	0.10
Ethylenediamine	Aliphatic/2 NH_2-	6.9	0.10
Ethylenediamine	Aliphatic/2 NH_2-	6.6	1.00
Citric	Aliphatic/3 COO-	6.4	0.10
Malonic	Aliphatic/2 COO-	5.6	1.00
Tartaric	Aliphatic/2 COO-	4.2	1.00

Table 3. Measured water chemistry during softwater acclimation/holding. Values are expressed as the mean ± standard deviation (n).

Test group	pH	Cu^{+2} (µg/l)	Ca^{+2} (mg/l)	Na^{+} (mg/l)	K^{+} (mg/l)
Experiment 1	6.46±0.26(23)	0.4±0.6(32)	5.1±0.7(39)	2.7±0.6(32)	2.3±2.0(25)
Experiment 2	6.22±0.36(16)	0.3±0.5(13)	4.6±0.4(13)	2.1±0.2(13)	-
Gill Cu Binding	6.28±0.21(10)	0.3±0.4(10)	4.9±0.4(10)	2.3±0.2(10)	-
Mucus Cu Binding	6.37±0.30(19)	0.4±0.6(19)	4.9±0.7(19)	2.6±0.6(19)	-

Table 4. Measured water chemistry during competition bioassays. Values are expressed as the five day mean ± standard deviation (n). The upper and lower portions of the table are for, respectively, (Experiment 1) and (Experiment 2) test sets.

Organic acid	pH	Cu^{+2} (μg/l)	Ca^{+2} (mg/l)	Na^{+} (mg/l)	K^{+} (mg/l)
Experiment 1					
NTA	6.23±0.31(5)	9.3±0.4(6)	4.7±0.5(6)	2.1±0.3(5)	2.2±2.6(3)
2,6-Pyridine	6.23±0.31(5)	9.7±0.7(6)	4.8±0.6(6)	2.1±0.3(5)	2.2±2.6(3)
Ethylenediamine	6.59±0.12(4)	8.3±0.3(7)	4.6±0.3(6)	2.4±0.4(6)	1.5±2.4(4)
Citric	5.88±0.36(5)	8.3±0.3(7)	4.4±0.1(6)	2.2±0.7(6)	4.1±0.6(4)
Malonic	6.40±0.29(4)	9.9±1.6(7)	4.8±0.2(7)	3.0±0.2(7)	1.9±2.3(7)
Tartaric	6.70±0.16(5)	9.3±0.5(5)	5.0±0.1(5)	13.7±1.5(5)	21.0±2.7(5)
Experiment 2					
Ethylenediamine	6.68±0.13(6)	8.8±0.7(5)	5.4±0.1(5)	3.2±0.1(5)	3.2±1.3(5)
Ethylenediamine	6.71±0.07(5)	9.2±0.6(5)	5.3±0.1(5)	3.2±0.1(5)	3.3±1.3(5)
Citric	6.62±0.24(9)	9.1±1.5(5)	5.4±0.1(4)	3.2±0.1(5)	3.1±1.4(4)
Malonic	6.47±0.20(6)	10.7±2.0(4)	4.5±0.2(4)	5.1±2.0(4)	1.8±1.9(4)
Tartaric	6.59±0.27(9)	9.6±1.2(4)	5.3±0.2(4)	17.5±1.4(4)	24.4±1.0(4)

(3.2.2) Results

As expected, 10 μg Cu/l alone (with no organic acids present) consistently lowered survival to $\leq$ 25% during the 5-day bioassays, whereas survival in clean water and organic-only controls was always $\geq$ 95% (Figure 7). In Experiment 1, the survival breakpoint indicates that the upper limit of the gill copper binding affinity corresponds to that of citric acid, with a log of the apparent binding affinity (ABA) of 6.4. Therefore, citric acid was the organic acid with the lowest copper binding affinity that could compete effectively with the fish gill for copper, and thus, prevent mortality.

In Experiment 2, the survival breakpoint was observed at a slightly higher value for log ABA, just above ethylenediamine (log ABA = 7.2) (Figure 7). The decreased survival exhibited by fish in the ethylenediamine-copper exposure in this experiment may have been due to a lower gill copper threshold concentration (as compared to the Experiment 1 fish) which must be reached before mortality begins.

As shown in Figure 8, gill copper accumulation decreased and survival increased as organic-copper binding affinity increased. Significant gill copper accumulation began when citric acid (log ABA 6.4) was used as the competing organic. At this point survival significantly decreased to 5%. Survival is also reduced to 90% (log ABA 7.2) and 78% (log ABA 6.8) with the higher binding strength organic acids. Since significant gill Cu accumulation began at the same point at which survival radically declined, measurement of gill Cu accumulation seems a viable alternative to identify a toxicologically relevant gill-copper binding affinity, as long as actual gill Cu concentrations are correlated to toxic effect. These data support the contention that the protective mode of the organic acids with higher copper binding affinities is to render the copper non-bioavailable, as the total copper concentration present would normally be lethal.

(3.3) Scatchard/NLR binding analyses

(3.3.1) Experimental approach and methods

Both the gill membrane and the external mucus coating have strong metal binding characteristics (Part and Lock 1983, Reid and McDonald 1991). Therefore, it is important to define both components since they function collectively to define the overall gill metal binding environment. The Scatchard equation (Scatchard 1949, Titeler 1981) converts non-linear, gill-copper or mucus-copper binding data to a linear form by expressing the ratio of bound to "free" copper as a function of bound copper, where the slope of the regression equation represents the binding constant (K) and the x-intercept equals the binding capacity (B_{max}) of the gill. When analyzing these data, we considered total copper equal to free copper (Cu^{+2}) for two interrelated reasons. Under our experimental conditions, at a pH of $\leq$6.5, $\geq$90% of the total copper exists as Cu^{+2}. Additionally, toxicity studies have found that both Cu^{+2} *and* $Cu(OH)_2$ are toxic, suggesting that either hydroxy species are also bioavailable, or, the hydroxy

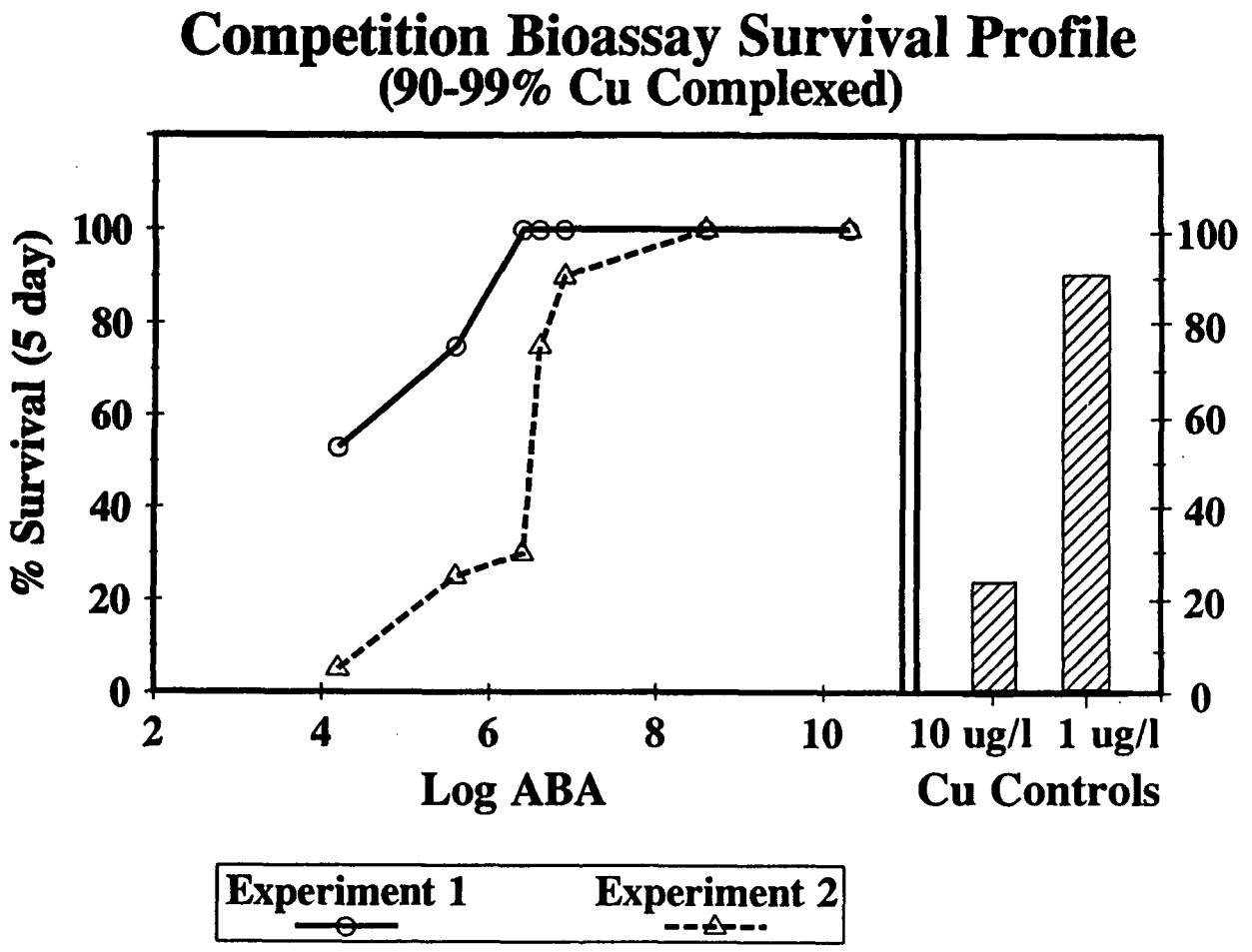


Figure 7. Competition Bioassay survival plots (Experiments 1 & 2). Right hand panel represents observed survival in the 10 μg Cu/l and 1 μg Cu/l copper-only controls. The 10 μg Cu/l control represents the maximum expected mortality and gill copper accumulation when a competing organic has a binding affinity significantly lower than the fish gill, while the 1 μg Cu/l control indicates the maximum expected survival and gill copper accumulation when a competing organic has a copper binding affinity significantly greater than the fish gill. Exposure chemistry is outlined in Tables 2 & 4.

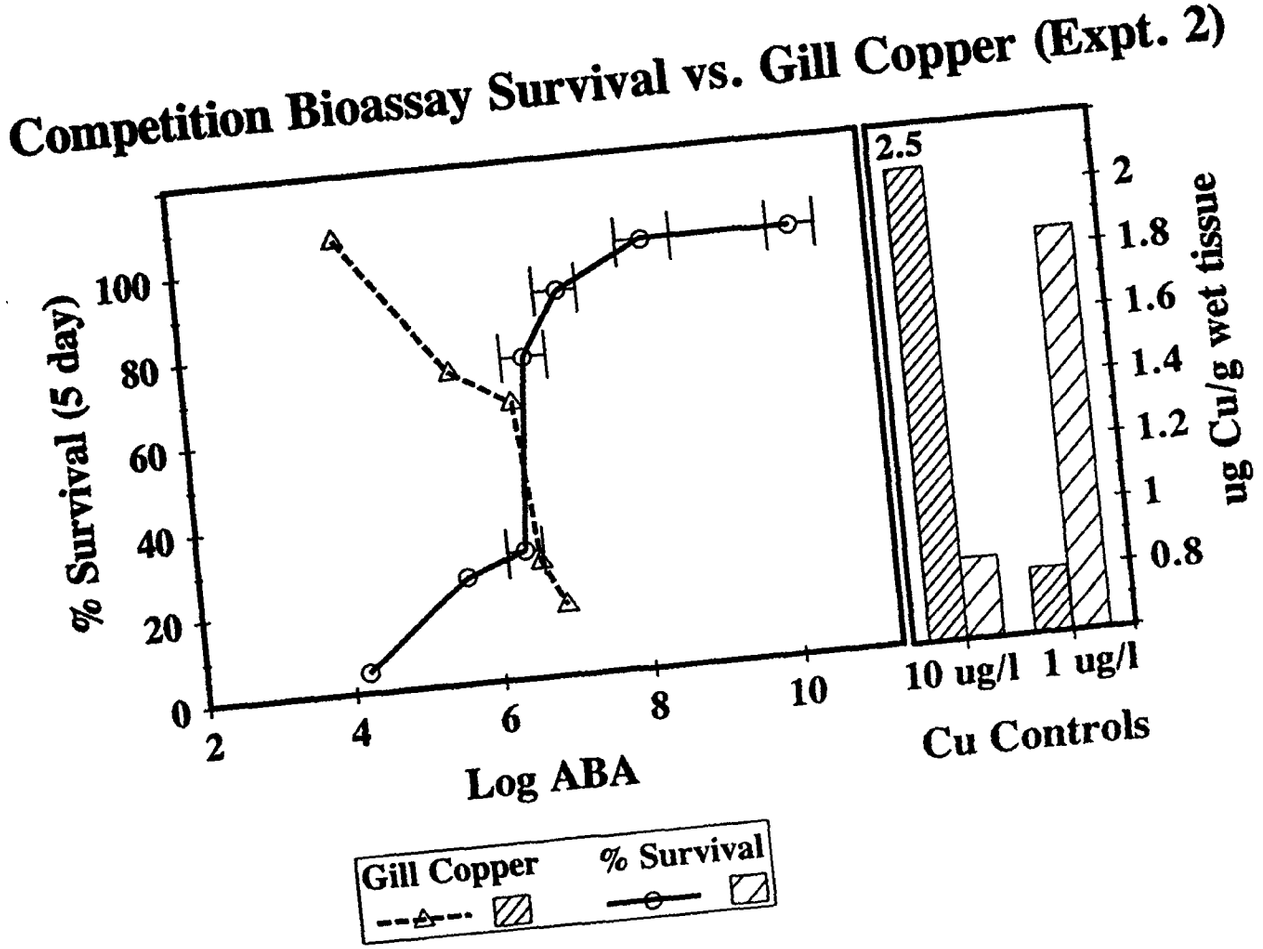


Figure 8. Percent survival and gill copper accumulation in the presence of organic acids of various copper binding affinities (Experiment 2 only). Right hand panel represents survival and gill copper concentrations in the 10 μg Cu/l and 1 μg Cu/l copper-only controls. The 10 μg Cu/l control represents the maximum expected mortality and gill copper accumulation when a competing organic has a binding affinity significantly lower than the fish gill, while the 1 μg Cu/l control indicates the maximum expected survival and gill copper accumulation when a competing organic has a copper binding affinity significantly greater than the fish gill.

species are converted to the bioavailable Cu^{+2} form within the gill microenvironment (Playle and Wood 1989a, 1989b, Randall et al. 1991). In either case, "free" copper at the gill microenvironment likely includes inorganic hyroxy species.

Any non-linearity within a Scatchard plot indicates that either multiple binding sites exist or there is cooperativity between various sites. To evaluate any non-linearity observed in a Scatchard plot, a Hill analysis may be used to establish the type of interaction. A Hill coefficient less than 1.0 indicates negative cooperativity and/or multiple binding sites, whereas a coefficient greater than one suggests positive cooperativity (Hill 1910, Titeler 1981).

For comparison purposes, we also used a non-linear regression (NLR) analysis that directly fits the non-linear gill-copper or mucus-copper binding data to either a one or two component equilibrium function, depending on the shape of the curve. This function is fit to standard geochemical equilibrium equations, where binding affinity and binding capacity are the key parameters. While this has long been a traditional geochemical approach to determining stability (binding) constants, it has not been favored among physiologists despite several apparent advantages over Scatchard analyses, such as greater statistical strength.

For the Scatchard/NLR analysis experiments, groups of 10 fish, either juvenile rainbow trout (RBT) or brook trout (BT -- obtained from the Wyoming Trout Ranch, Cody, WY), were exposed for 24 hours to one of 11 free Cu^{+2} ion concentrations ranging from 0.5 to 20.0 μg/l (0.008 to 0.315 μmol/l), under identical water chemistry conditions to those used in the competition bioassays, but without organic acids. Gills were then excised after 24 hours and processed as above. *Daphnia magna* were tested using a similar procedure, but were acclimated to water representative of their usual habitat, then tested in the same soft water used above. Fish mucus was evaluated similarly, but rather than an in-situ procedure, the mucus was removed from the fish then analyzed using bench-level approaches. Since gill mucus is difficult to obtain in sufficient quantities for analysis, body mucus was collected instead. A study by Part and Lock (1983) showed that body mucus and gill mucus have identical protein composition according to polyacrylamide gel electrophoresis banding patterns. Since protein is considered to be a primary component responsible for metal binding, body mucus was considered a suitable gill mucus analog.

All concentrations of copper in water exposures and digested tissues were determined with graphite furnace atomic adsorption spectrophotometry (GFAAS).

(3.3.2) Results

Gill copper accumulation in RBT and BT exposed to a series of free Cu^{2+} concentrations (in the absence of any competing organics) for 24 hours is shown in Figure 9, and the associated Scatchard binding plots are presented in Figure 10. Again, the slope of a Scatchard plot reflects the binding constant (K), while the abscissa intercept represents the maximum concentration of metal bound by the gill

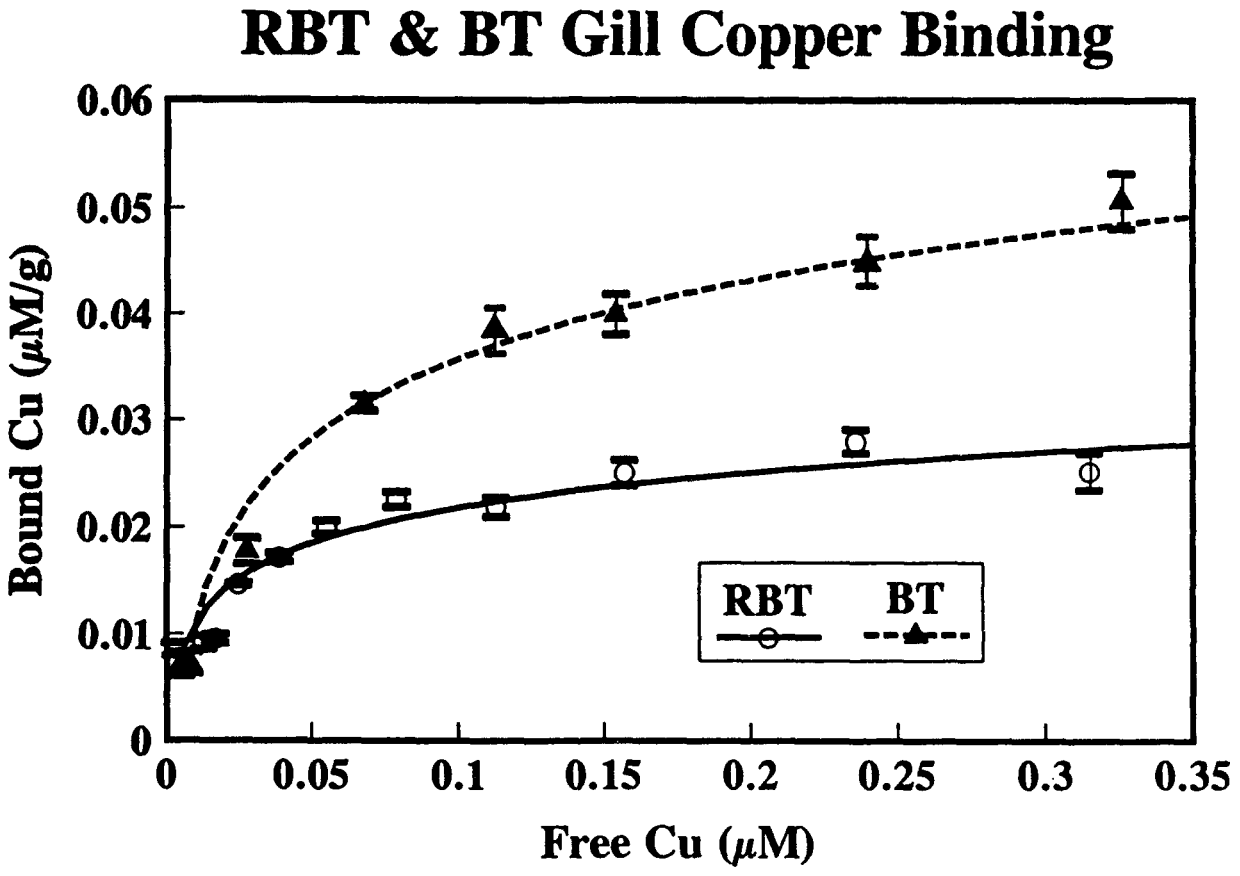


Figure 9. Gill copper accumulation on the gills of Rainbow and Brook trout in μM Cu/g wet tissue plotted against free Cu^{+2} ion concentrations. Exposure duration is 24 hours. Exposure water chemistry is identical to that used in competition bioassays (Tables 2 & 4), but with no competing organic acids present.

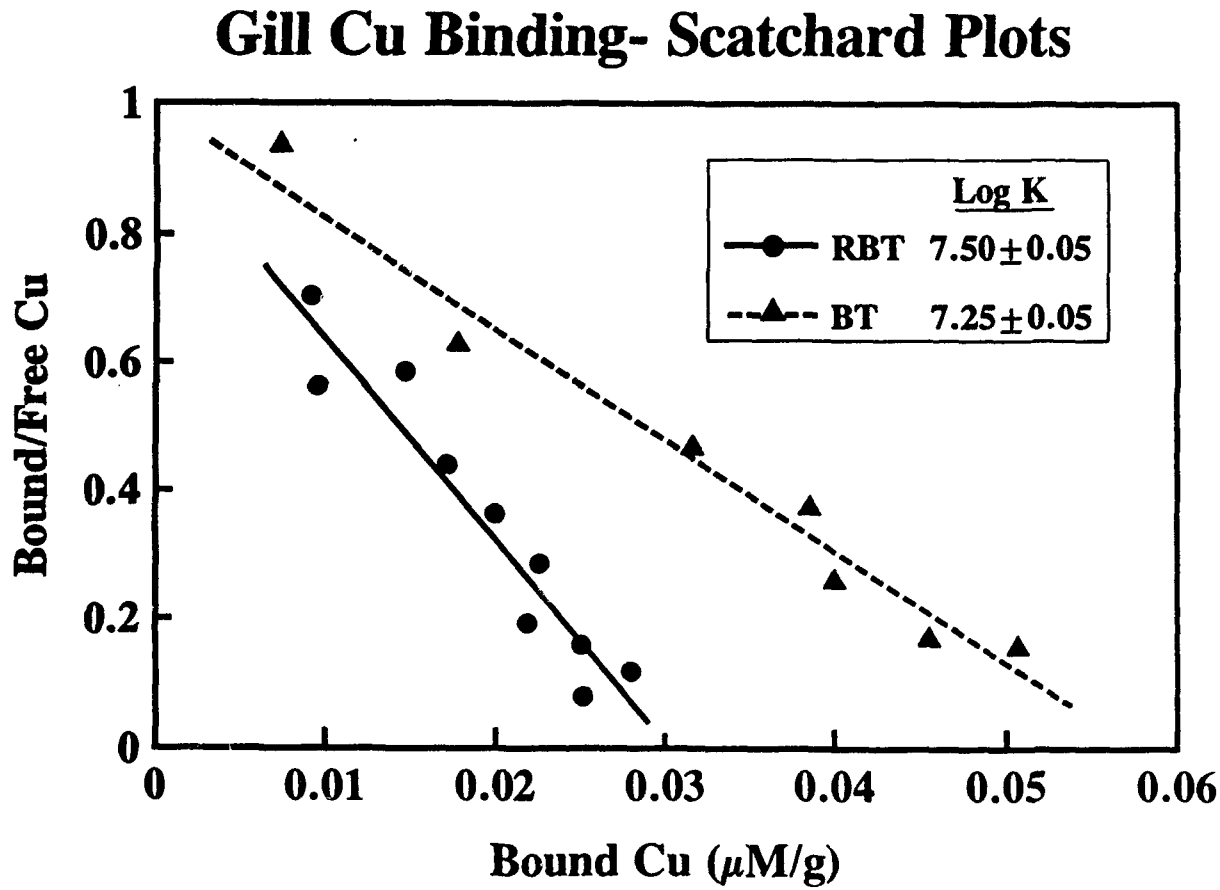


Figure 10. Combined Scatchard Plots of gill Cu binding data from Figure 9 showing both Rainbow and Brook trout regressions. The Rainbow trout regression equation is B/F = B(-31.91) + 0.97 (r^2 = 0.915), log K (pK_{Cu}) = 7.50, B_{max} = 0.030 μM/g. The Brook trout equation is: B/F = B(-17.70) + 1.01 (r^2 = 0.970), log K (pK_{Cu}) = 7.25, B_{max} = 0.060 μM/g. Regressions are significantly different ($p < 0.001$).

(Scatchard 1949). The RBT gill binding constant resolved using this Scatchard re-plot of the copper accumulation data was log K = 7.50 ± 0.05, in close agreement with the apparent binding affinities (ABA) determined with the competition bioassays (log ABA = 7.2 or less). The B_{max} value from the Scatchard plot for RBT was 0.030 μM/g; however, this value should be interpreted with caution, because with copper it is highly dependent on the exposure duration. The longer the exposure, the higher the B_{max} will be due to continued internalization of copper within the gill. The Hill plot (Figure 11) coefficient of 0.992 for RBT was not significantly different from 1.0, indicating there is no cooperativity between gill binding sites, and only one type, or one dominant class of binding sites, under the conditions tested here (Hill 1910). For the BT Scatchard analysis shown in Figure 10, the log K was 7.25 ± 0.05, the B_{max} was 0.060 μM/g, and the Hill coefficient was 1.0. The BT gill Cu binding affinity was significantly lower ($p<0.001$) than the RBT gill Cu binding affinity, as might be expected given the lower sensitivity of brook trout to Cu toxicity.

The NLR analysis yielded similar results to the Scatchard analysis of gill-copper binding. A one-component model fit best, where:

$$\text{Bound} = \text{Free}*((10^{\text{Log K}})*B_{max}/1+(10^{\text{Log K}})*\text{Free})).$$

The RBT log ABA was 7.56 ± 0.06, B_{max} = 0.029 ± 0.001 μmol/g, r^2 = 0.966 and for BT, log ABA 7.14 ± 0.08, B_{max} = 0.063 ± 0.004 μmol/g, r^2 = 0.989. This further supports single, dominant copper-binding ligand classification of the gill surface, and also strongly suggests the gill may be interpreted as a geochemically definable surface, at least under the conditions examined here.

Unlike the gills, the rainbow trout mucus-copper binding Scatchard plot was distinctly non-linear, indicating multiple sites or negative cooperativity. For mucus-copper binding constants, the log K_1 values = 6.9 to 7.7. The *Daphnia magna* copper binding Scatchard plots were also non-linear, with the log K_1 values = 6.6 to 8.1. For *Daphnia magna*, the two component binding profile may reflect two independent locations for copper binding. But most importantly, the copper-binding constants determined here for both fish mucus and a completely different Class of aquatic organism all fall within the range of constants determined for RBT and BT gills.

(3.4) Discussion

(3.4.1) Competition bioassay and Scatchard/NLR methods

By using both competition bioassays, based on toxicity, and Scatchard/NLR analyses, based on copper residue accumulation, to estimate the apparent binding affinity (log ABA) of gill for copper, we have provided an approach for the determination of copper bioavailability that has both geochemical *and* toxicological significance. But to properly evaluate the competition bioassay results, the survival breakpoint and the beginning of gill copper accumulation (see Figure 8) must be interpreted carefully. For instance, removal of a small amount of copper by the gill

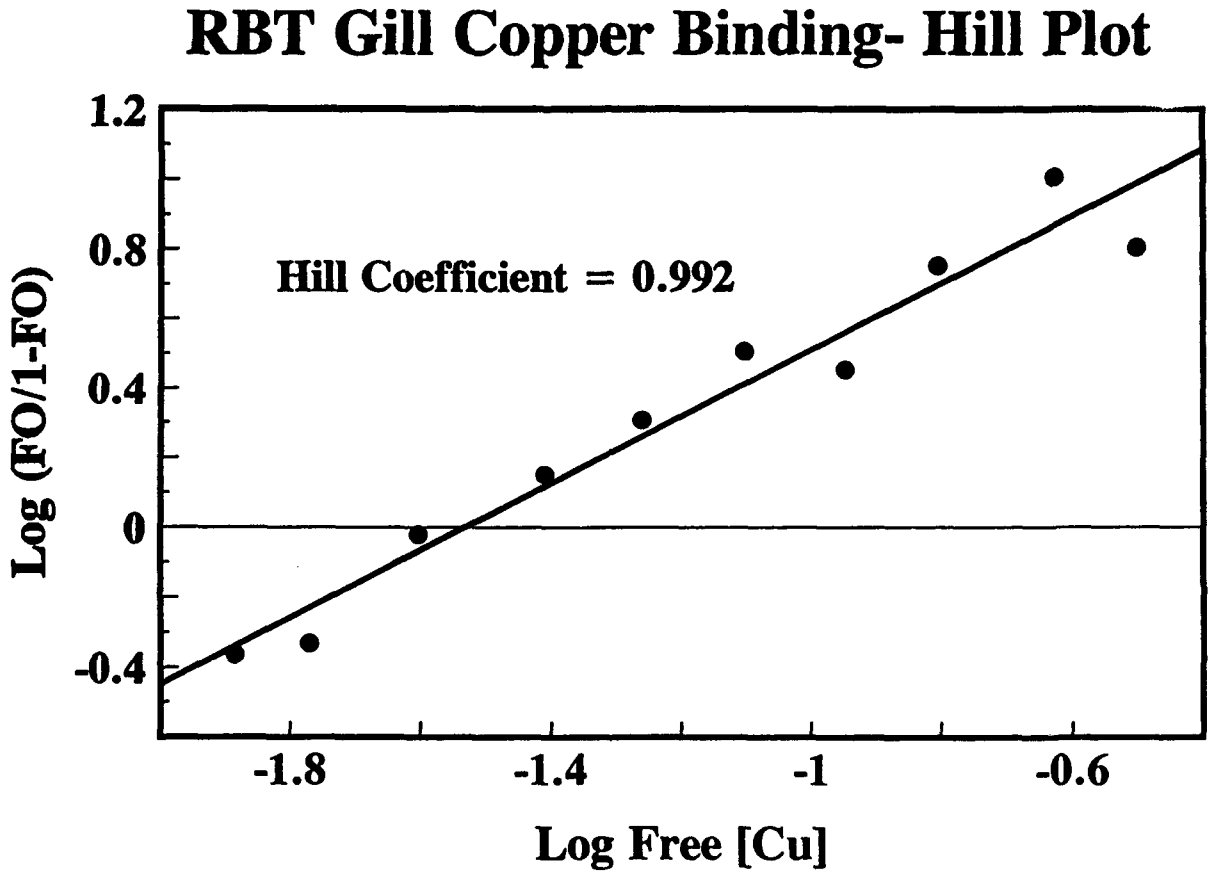


Figure 11. Hill Plot of Rainbow trout gill Cu binding data from Figure 9. The regression is: Log (% Sites Occupied/1-% Sites Occupied) = log [Cu](0.992) + 1.5148, where the slope (0.992) corresponds to the "Hill coefficient". Coefficients lower than 1.0 indicate multiple binding sites and/or negative cooperativity between sites, while coefficients greater than 1.0 indicate positive cooperativity among binding sites.

would be expected even if the gill binding affinity was still weaker than the competing organic acid ligand, based on equilibrium dynamics alone (Allison et al. 1991). Theoretically, then, only a significant decrease in survival (and increase in gill copper) should define the point at which the gill binding strength is greater than or equal to that of the competing organic acid. Therefore, technically, only a gill binding affinity *range* can be defined. Yet from a practical perspective of defining bioavailable metal, only the upper limit need be considered, because this would provide a conservative estimate of the gill binding affinity, and thus, a conservative estimate of bioavailable metal. In support of this interpretation, consider the close agreement between our binding affinities determined by the competition bioassays and by the Scatchard/NLR plots (log ABA 6.4-7.2 vs. 7.1-7.6, respectively).

An additional consideration of the approach used in this study must be the applicability of the apparent binding affinity determined in this study to the variety of "real" natural water chemistry, given the controlled, and relatively simple water quality conditions used in these tests. Because an experimentally determined metal binding affinity can be modified by the chemical constituents present during the test, the binding constant so determined will be an "apparent" constant (ie. "conditional stability constant", using geochemical terms), and not an absolute one. But the current literature on water chemistry-toxicity relationships supports our hypothesis that our *apparent* estimate is quite near an "absolute" one at circumneutral pH's. In terms of competition reactions at the gill, toxicity studies by Lauren and McDonald (1985, 1986) and a preliminary study at this laboratory (unpublished data) have demonstrated that Ca is a poor competitor with Cu for binding sites when fish are exposed to copper at the *same* ambient calcium concentration present during acclimation (see Objective 2). Moreover, Playle and Dixon's (1994a) study of fathead minnows found that the gill Ca^{+2} binding constant is between log K 3.4 and 5.0, and Pagenkopf (1983) estimated that the value for rainbow trout is between 3.3 and 4.1, all significantly lower than the gill copper binding affinity of log K 7.4. As a result, it is likely that the 5 mg Ca/l used in this study will also have a minor effect on the binding affinity determination. Therefore, the "apparent" binding affinity determined here is likely close to the absolute affinity for this pH range. It is also likely that the cation exchange resin ultimately selected will exhibit similar selectivity, because many resins we reviewed in the literature have far greater binding affinities for heavy metals than for Ca^{+2} (Rhom and Haas 1991, Bio-Rad 1992). Similarly, other ions, such as K^{+}, Na^{+}, Mn^{+2} or Mg^{+2} are often present together with Cu^{+2}. Depending on their concentrations, they may compete to varying degrees with copper for gill ligands, and thus change the apparent gill-copper binding affinity. However, as with Ca^{+2}, most biological surfaces (as well as cation exchange resins) have a significantly greater affinity for copper over these other ions (Mann 1990, Volesky 1990, Kuyucak and Volesky 1990, Reid 1991, Rhom and Haas 1991, Bio-Rad 1992).

Decreases in pH, however, can reduce copper toxicity (Cusimano et al. 1986) and copper uptake (Lauren and McDonald 1986), and thus, will potentially change the *apparent* gill copper binding affinity. Several studies estimate the average cell membrane/gill surface pKa range from 4.0-5.4, as determined by surface binding

analyses or estimation from low pH toxicity test results (Seimiya and Ohki 1973, Pagenkopf 1983, Cusimano et al. 1986, Reid 1989, Playle and Dixon 1994a). Given these constants, at a pH of 6.5, a high percentage of gill binding sites will be deprotonated and anionic, and thus most available to copper binding. As a result, the apparent copper binding constant of the gill determined under these conditions will reflect a highly sensitive state of the gill toward copper binding and a near maximum gill-copper binding affinity. Moreover, this suggests H^+ may not be able to compete effectively for copper binding sites when present at equinormal concentrations to Cu (i.e., at circumneutral pH's 6.0-8.0 ($[H^+]$ = 1-0.001 ueq/l ≈ 32-0.03 μg Cu/l)). Only when the pH decreases and H^+ concentrations rise enough to allow H^+ to compete by mass action will the *apparent* copper binding affinity be changed. This competition will need to be quantified before the cation-exchange technique can be applied to low pH waters.

Lastly, other common toxic metals, such as Pb^{+2}, Cd^{+2}, and Zn^{+2}, and generally non-toxic metals such as Fe^{+2} and Mn^{+2} are often present together with Cu^{+2}, and as with H^+, can compete for gill binding sites and thus alter the apparent copper binding affinity. Therefore, any analytical separation tool such as geochemical models or a cation-exchange resin "artificial gill" must simulate these interacting binding affinities. This will be the subject of continuing research at this laboratory on both gill and cation-exchange resin binding properties.

(3.4.2) Comparison to other methods

The copper binding affinities for rainbow trout gills, for brook trout gills, for rainbow trout gill mucus, and for *Daphnia magna*, determined in this study, agree well with copper binding affinities for other aquatic organisms reported in the literature (Table 5). For instance, the binding affinity determined for fathead minnow (*Pimephales promelas*) gills by Playle and Dixon (1994a) (log K = 7.4) is very close to the values we determined for rainbow and brook trout, even though fathead minnows are less sensitive to copper toxicity (USEPA 1985). This suggests that a single gill copper binding affinity may be common among various species of fish, perhaps a reflection of fundamental gill-membrane characteristics. This is not surprising since the binding properties of the gill are constrained by basic physiological needs for O_2 transfer, and binding and absorption of critical osmoregulatory ions, such as Ca^{+2} or Na^{+1}. *Daphnia magna* also have copper binding constants similar to fish gills, and metal binding studies performed with the alga, *Chlamydomonas rheinhardii*, show their body surface has a two component copper binding profile, with binding constants of log K_1 = 8.0 and log K_2 = 6.5 (at pH 6.5), and capacities of 10-40 μmoles/g (Xue et al. 1988). And lastly, a study by Rudd (1984) found that the copper binding constant of the bacteria *Klebsiella aerogenes* cell wall expolymers was log K = 7.9 (Mann 1990). While these species are obviously very different from fish, their copper binding constants are amazingly similar to that of fish gills, and the capacities are approximately within an order of magnitude. Therefore, it is intriguing to postulate that differences observed in toxicity among species may be due in part to differences

Table 5. Summary of physiologically relevant copper binding affinities and capacities determined by this and other studies.

Species	Method	ABA (Log K)	B_{max} (μmol/g)	Reference
Rainbow trout	Competition bioassay	6.4-7.2	-	This study
Rainbow trout	Scatchard/Non-linear regression	7.5-7.6	0.03	This study
Rainbow trout	Isolated "gill dip"; radiosotope based kinetic examination	2.4	0.93	Reid & McDonald, 1991
Rainbow trout-"gill" mucus	Equlibrium dialysis Satchard/Non-linear regression	6.9-7.7	-	This study
Brook trout	Scatchard/Non-linear regression	7.1-7.2	0.06	This study
Fathead minnows	Scatchard	7.4	0.002	Playle & Dixon, 1994
Daphnia magna (Invertebrate)	Scatchard	6.6-8.1	5.00	This study
Chlamydomonas rheinhardii (Algae)	Scatchard	6.5-8.1	25.0	Xue et al., 1988
Klebsiella aerogenes (Bacteria)	Scatchard	7.9	-	Mann, 1990

in repair, compensation, distribution, and elimination mechanisms, rather than major differences in metal adsorption.

Interestingly, however, the gill copper binding affinity determined in this study does disagree quite significantly with the value determined by Reid and McDonald (1991) for rainbow trout gills (Table 5). They concluded that the rainbow trout gill copper binding affinity was log K = 2.7, which is significantly lower than the value from this study and the Playle and Dixon (1994a) value for fathead minnows. Their method, though, involved exposure of individual, excised, and EDTA rinsed gill arches, rather than live fish, to radiometal solutions. This procedure may well have distorted normal gill structure and physiology by opening cell junctions, by disrupting cell membranes, by altering blood flow and hormone concentrations, by inducing a proliferation of a thin mucus coat on the binding surface, which could both slow binding kinetics and inhibit contact of the metals with the gill (Part and Lock 1983, Powell et al. 1992), or by removal of a mucus coat which may facilitate ion binding by ion-exchange mechanisms and/or unstirred solution layers (Part and Lock 1983, Shepard 1992).

An explanation for this apparent discrepancy in binding affinities relies on the definition of gill copper binding affinity chosen. The exposure time used in the Reid and McDonald (1991) study was only five minutes, versus the 24-hour exposure time of this study, and the 3-hour exposure used by Playle and Dixon (1994a). As they point out, this short exposure time would allow external surface binding only, whereas a longer exposure time would allow both surface binding and internalization (Reid and McDonald 1991, Lauren and McDonald 1987). Therefore, the low binding affinity reported in their study may reflect surface binding only, and not the stronger affinity of internal ligands and/or surfaces. Lauren and McDonald (1987) propose that the basolateral surface of the gill, and specifically, the Na/K ATPase pump, is a primary site of copper toxicity, more so than the gill surface or tight junctions. From a toxicological perspective, then, the internalization binding affinities determined in this study's and Playle and Dixon's (1994a) studies may be more appropriate for defining bioavailable copper.

(3.4.3) Environmental significance

The copper binding constants determined in this and Playle and Dixon's (1994a) studies suggest some forms of DOC-bound copper may be available to fish, because Cu-DOC binding constants can range from 4 to 15 (log ABA), with average values for humic acids of 4.9-6.8 (Guy and Chakabarti 1976, Leckie and Davis 1979, Giesy and Alberts 1989, Nor and Cheng 1989). Because these humic acid values are lower than that of the fish gill, the protective effect of DOC must be a function of its high metal binding capacity and aqueous concentration, rather than its copper binding affinity alone, and additional DOC components from humic acids which contribute to its overall copper binding properties. Giesy and Alberts (1989) also note that the Cu-humic acid complex strength is greater than similar complexes formed with other toxic

metals such as Pb^{+2}, Cd^{+2}, or Zn^{+2}. This suggests that the gill may be able to remove other metals from natural humic materials more readily than copper.

(3.4.4) Possible future methods -- geochemical modeling

Another common approach to quantifying bioavailable metal is to expand or create a geochemical speciation model to include experimentally determined metal binding constants and capacities for both biotic (e.g., fish gills) and abiotic (e.g., humic materials) components. While geochemical speciation models of aquatic systems are quite abundant, they rarely include both components. However, two studies have addressed the missing biotic component. Pagenkopf (1983) used empirical toxicity-water chemistry relationships to determine fish gill metal binding constants, then inserted them into his "Gill Surface Interaction Model" (GSIM) which allowed predictions of not only inorganic speciation, but gill copper concentrations as well. However, actual gill metal binding characteristics were never measured and dissolved organic carbon (DOC) was not included in the model. Playle and Dixon (1994a) measured various gill-metal constants for fathead minnows by analyzing gill-metal accumulation data, although toxicity was never *directly* correlated to these constants. By inserting these constants and experimentally determined metal-DOC constants into the geochemical speciation program MINEQL (Schecher 1991) they could accurately predict gill copper accumulation for some natural waters.

Both approaches not only quantify the copper forms present, but also estimate the amount of metal bound to the fish gill. This second component represents the bioavailable metal fraction. But even though modelling does begin to account for biological influences on metal speciation, and thus bioavailability, it is seriously constrained by its heavy dependence on accurate and appropriate binding constants, as well as accurate and thorough quantification of *all* the potentially interacting components present, including the ligands of sensitive resident organisms, dissolved and suspended solids, and DOC (Neubecker and Allen 1983). This, in turn, depends on a thorough understanding of the structure and function of all components, and with the gill, identification of a binding constant that is toxicologically relevant. Meeting all these constraints is time consuming, and it is often difficult and quite expensive to quantify all the chemical parameters necessary for geochemical modeling. So although geochemical speciation models may provide adequate estimates of bioavailable metal in some circumstances, their widespread practical applicability is debatable at this time.

(3.4.5) Possible future methods -- cation exchange chromatography

Defining the toxicologically relevant copper binding affinities of various biological ligands such as the fish gill is critical to accurately measuring bioavailable metal. Therefore, short of site-specific toxicity evaluations, an "artificial biological ligand" in the form of a cation-exchange resin could meet this need. Because the competition bioassays, our Scatchard analysis of gill copper binding, and Playle and Dixon's (1994a) fathead minnow analysis all have upper limits for gill copper binding

affinities of 6.4-7.6 (log ABA), we conclude that 7.6 would be the most appropriate and toxicologically relevant constant. But even if we assume our log ABA estimate is an entire log unit low, when we consider this logarithmic scale, an estimate of log ABA 8.0 would predict that there is still 100x less bioavailable copper than that predicted using log ABA 10.0+, which is a common ABA for several currently available cation exchange resins (see Section b.4, below). Additionally, the Hill coefficients and NLR fit for both rainbow and brook trout Scatchard analyses suggest the toxicologically significant metal binding environment may be straightforward enough to simulate with a cation exchange resin. A cation exchange resin with this copper binding affinity could, therefore, be a simple analytical method to determine the fraction of total metal available to fish. Its success is not dependent on difficult and time-consuming analyses of water quality parameters, nor on a vast array of geochemical binding constants necessary to describe the numerous chemical species possible in an aquatic environment. The amount of metal removed from a water sample expressed as a fraction of total metal represents the bioavailable component.

(4) Cation Exchange Chromatography Procedures

(4.1) Commercial cation-exchange resin evaluations

Experiments to date and a review of the current literature indicate most commercially available ion exchange resins have apparent copper binding affinities far greater than that of the fish gill. Three cation exchange resins were evaluated more extensively for copper binding characteristics (Rhom and Haas Amberlite IR120+, Rhom and Haas DP-1, and Bio-Rad CMBio-Gel) using a method analogous to the competition bioassays (Rhom and Haas 1991, Bio-Rad 1992). The Amberlite IR-120+, is considered a "strong" cation exchanger due to its sulfonic acid functional group, whereas DP-1 and CM-BioGel are considered "weak" exchangers, due to their methacrylic-carboxylic acid and agarose based weak carboxylic acid functional groups, respectively. Both IR-120+ and DP-1 resins have extremely high cation exchange capacities ($\approx$5 meq/ml), as compared to the ambient copper concentration present during this study (0.310 μeq/l), which will increase their overall *apparent* copper binding affinity (as determined with our experimental approach). In contrast, while the CM-BioGel resin is also considered a "weak" exchanger, it has a significantly lower cation exchange capacity (0.5 meq/ml). This resin is designed to separate proteins in biological systems, and as such would more likely have a lower binding capacity and, potentially, an apparent affinity closer to that of the gill surface.

These apparent resin-metal binding strengths can be modified by varying both eluent flow rates and the counter-ion present. However, previous experiments performed with these resins and aluminum-organic species (Fernandez 1994) suggested changing counter-ion would be a more effective method to vary binding strength than modifying flow-rate. Consequently, the typical experimental procedure used to evaluate the apparent copper binding affinity of IR-120+ and DP-1 resin/counter-ion combination involved the following steps: (1) prepare an organic-copper solution identical to that used in the competition bioassays; (2) convert the

resin to the desired counter-ion (either Na, Ca, Fe, Al, or H); (3) fill a 1 x 10 cm column with resin; (4) equilibrate the column to control water; (5) pump the organic-copper solution through the column, and collect an eluent sample after approximately 100 mls has passed; (6) analyze the eluent for copper by GFAAS. The presence of copper in the eluent indicates the competing organic had an apparent binding affinity greater than that of the column.

The procedure used to evaluate the CM-BioGel resin was similar to that used above, but eluent samples were collected serially over the course of elution, rather than only once. This method allowed us to determine the copper partitioning within the column as equilibrium was approached, which is a more appropriate simulation of events occurring on the fish gill.

It is evident from Figure 12 that only the organic acid with the strongest binding affinity used in the competition bioassays (NTA; log ABA = 10.3), could effectively compete with any of the three resins tested. Counter-ion did not seem to have a major influence, although use of Ca^{+2} resulted in a slightly lower affinity with the IR-120+ resin. Considering the logarithmic binding constant scale, this study's gill copper binding affinity is vastly lower than the resin binding affinity of ABA 10.3. Therefore, use of such a column can greatly overestimate the true bioavailable metal fraction present.

Because both binding affinity as well as capacity will determine the "apparent" binding affinity of a gill or ion exchange column, we continued experiments with columns containing only one tenth of the resin used in the above experiments. Unfortunately, the results were similar to those described above, suggesting the high apparent binding affinity exhibited by these resins is due to either a high binding constant, and not simply an overwhelming binding capacity, or a capacity many orders of magnitude greater than that of the gill.

(4.2) Custom synthesized cation-exchange resin evaluations

Most cation exchange resins that are available commercially are designed to efficiently remove a cation of interest, rather than selectively remove a particular form of the cation (i.e., an "all or nothing" action). Usually this efficiency of removal is facilitated by extremely high exchange capacities, often many orders of magnitude greater than biological surfaces of similar size. Therefore, our synthesis approach was to create a low capacity resin that simulates the capacity as well as the copper binding affinity of the fish gill, using silane coupling agent chemistry techniques (Morrall and Leyden 1985, Arkles 1991, Turkova 1993). Here, chemically modified organic ligands, such as ethylenediamine, are covalently bonded to glass beads, where the larger the bead size used, the lower the resulting binding capacity per unit weight. By varying glass bead size and organic ligands, for example, we can tailor a resin to our specific needs.

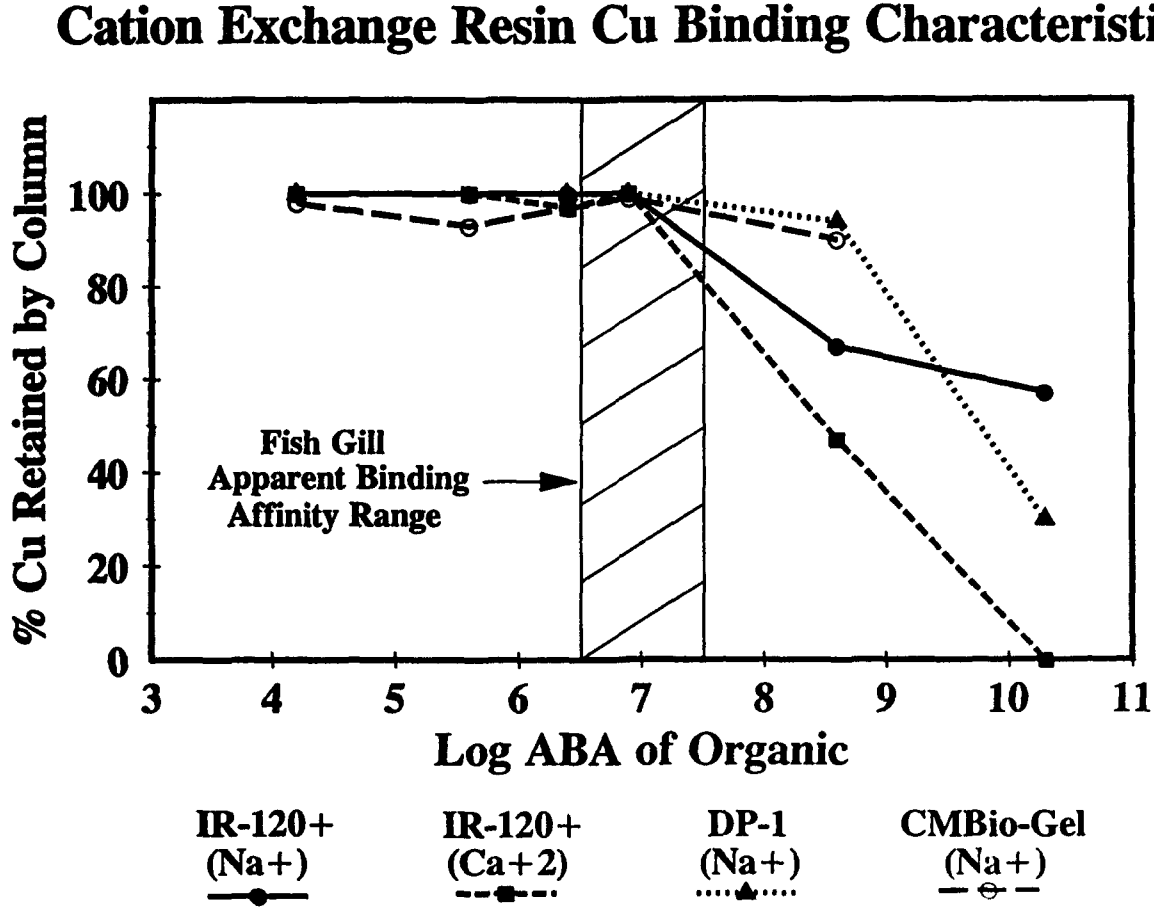


Figure 12. Cation-exchange resin Cu binding characteristics for 3 different resins and 4 different counterions. Retention of Cu by the column indicates the resin has an apparent binding affinity (ie. an "operationally defined" affinity influenced by column size, flow rate, and resin binding affinities and capacities) greater than the organic ligand. The open bar represents the apparent gill binding affinity range as determined in this study.

Three resin forms were synthesized by covalently bonding an organosilane derivative of either ethylenediamine (EDA), malonic acid, or urea to 250 μm acid cleaned glass beads. In a free, underivitized form, EDA was the strongest copper binding ligand of the three (apparent log K_{cu} = 7) while malonic acid was the weakest (apparent log K_{cu} = 5.6). The urea- or malonic-silanes were added to 25mls of a 95% ethanol-5% water solution to achieve a final organo-silane concentration of 2% v/v. Solution pH was adjusted to 5.0 with acetic acid, then stirred for 5 minutes to allow hydrolysis and silanol formation. Twenty-five grams of glass beads were then added, followed by an additional three minutes of constant stirring. The organo-silane/ethanol solution was then decanted away and the beads were washed twice with 95% ethanol, then allowed to air dry for 24 hours. The EDA-silane resin synthesis was identical, but the acetic acid pH adjustment step was omitted to avoid excessive polymerization of the organo-silane before deposition.

Following synthesis and drying, resins were converted to the Na^+ counterion form, and stored in deionized water. Resin binding strength was evaluated using a method analogous to the Scatchard/NLR experiments, rather than the competition assay method used previously to evaluate the commercial resins. This approach was adopted for several geochemical reasons, which will not be detailed here. We have found, however, that this approach is more realistic in terms of "real world" interactions between fish gill ligands and their environment than a column method can simulate. For this method, 0.50 ± 0.01g of resin was added to each of ten different beakers containing 250 mls pH 6.5 deionized water, 5 mg Ca/l, 2 mg Na/l, and one of 7 free Cu^{+2} ion concentrations ranging from 0 to 300 μg Cu/l. Untreated glass beads were used as a control, although some non-specific binding was expected due to negatively charged silicate functionalities. After one hour, the resin was removed and washed in 15 mls of 10% HNO_3 to remove any bound copper. The HNO_3/Cu solutions and pre-resin beaker solutions were then analyzed for copper using GFAAS to determine bound and free copper, respectively.

As expected, the control beads had some affinity for copper (Log K_{cu} = 6.1), but an extremely low capacity (0.006 μmol/g). Addition of an organo-silane increased the capacity (0.016 μmol/g), however, the log K_{cu} value remained essentially constant among the three organo-silanes chosen, with no appreciable difference from the control (Figure 13). The commercial resin Amberlite IR-120+ was also evaluated as a comparison and method check. The log K_{cu} was slightly higher (6.8), but the capacity was far greater than any of the synthesized resins ($\approx$0.5 μmol/g), although still far lower than that stated by the manufacturer (500 μmol/g). This low binding capacity is not surprising, however, since the experimental design used here was not really appropriate to evaluate the binding capacity (B_{max}) of such a high capacity resin. The B_{max} value reported is a tenuous extrapolation since resin saturation was never approached. Nonetheless, IR-120+ had a significantly higher binding capacity, and thus *apparent* affinity, than our synthesized resins, as suspected earlier in our competition evaluations (Figure 12). Since we were clearly able to distinguish the IR-120+ resin from the low-capacity organo-silane resins, our evaluation method seems appropriate.

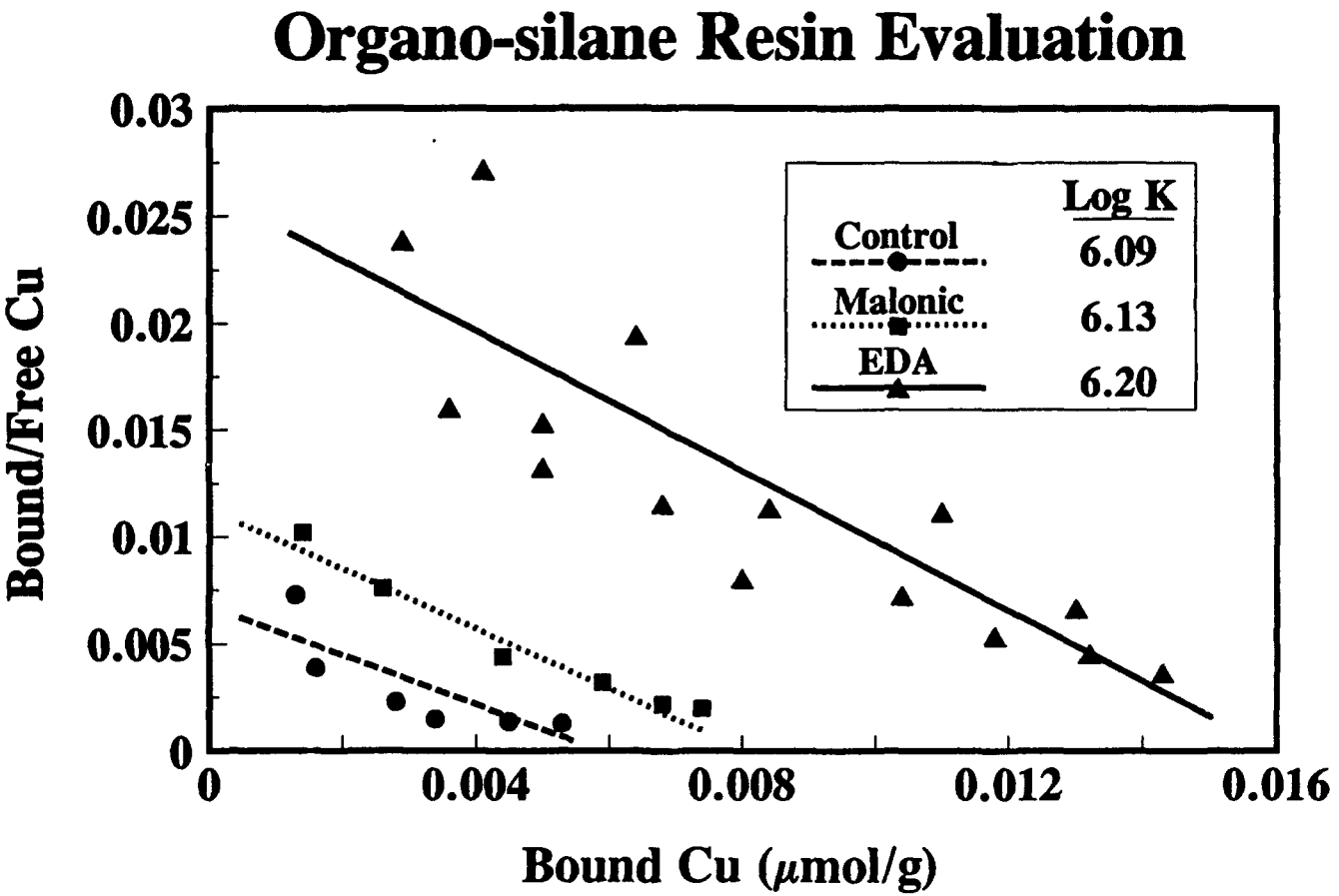


Figure 13. Scatchard Plot linearizations of copper binding by two synthesized organo-silane cation-exchange resins and a glass-bead "control", plotted as Bound Cu (μM/g)/Free Cu (μM) versus Bound Cu (μM). Log K values are derived from the slope of the Scatchard regressions. Resins were evaluated with the same exposure water chemistry as that used in the competition bioassays and Scatchard/NLR analyses (Tables 2 & 4), but with no competing organic acids present.

(4.3) Discussion

Definition of the toxicologically relevant copper binding affinities of various biological ligands, such as the fish gill, is a critical step toward accurate measurement of bioavailable metal concentrations. Therefore, aside from site-specific toxicity evaluations, an artificial biological ligand in the form of a cation exchange resin could meet this need. Because the competition bioassays, our Scatchard analysis of gill copper binding, and Playle and Dixon's (1994a) fathead minnow analysis all have upper-limit gill copper binding affinities of 6.4-7.6 (log ABA), we conclude that 7.6 would be the most appropriate and toxicologically relevant binding constant. Additionally, the linearity of the Scatchard plots, the near unity Hill coefficient, and the high correlation of the single component NLR fit suggest that there is either a single gill copper binding component, or more realistically, considering the complexity of the gill binding environment, a single dominant component or several components with similar binding properties that function collectively to define the binding environment. This suggests that the toxicologically significant metal binding environment may be straightforward enough to simulate with either a cation exchange resin, or alternatively, with a geochemical model.

Clearly, ambient Ca^{+2} and Mg^{+2} concentrations, pH, other metals such as Fe^{+2} and Mn^{+2}, and other trace metals such as Al^{+3}, Zn^{+2}, Pb^{+2}, and Cd^{+2} are often present with Cu^{+2}, and can compete for gill binding sites and thus alter the apparent copper binding affinity. Therefore, any analytical separation tool such as a geochemical model or a cation exchange resin "artificial gill" must also simulate these interacting binding affinities. For some ions, such as Ca^{+2} and Mg^{+2}, biological surfaces as well as carboxylic cation-exchange functionalities have a significantly greater affinity for Cu^{+2} over these ions (Mann 1990, Volesky 1990, Kuyucak and Volesky 1990, Reid 1991, Rhom and Haas 1991, Bio-Rad 1992), so they should not significantly alter the apparent gill-copper or cation-exchange resin copper binding affinity. Other metals and H^{+} ($pH < 6.0$) can be significant competitors though, and therefore these competition reactions would need to be simulated. The selectivity of many carboxylic ion exchange functionalities follows the order:

$$H^{+1} > Hg^{+2} > Cu^{+2} > > Pb^{+2} = Fe^{+3} > Al^{+3} > Zn^{+2} > Cd^{+2} =$$

$$Co^{+2} > Fe^{+2} > Mn^{+2} > Ca^{+2} = Mg^{+2} > > > Na^{+1}$$

which, with the exception of Cd^{+2}, corresponds to the toxicities and empirically observed competition reactions among these metals (BioRad 1992, Rhom and Haas 1991, Sorensen 1991). Assuming toxicity is directly related to gill-metal binding, this cation-exchange selectivity series would seem to agree with the expected gill selectivity series. Likewise, we might expect a *synthesized* cation exchange resin with carboxylic acid functional groups to exhibit similar selectivity.

Currently, geochemical modeling is the most popular method to quantify bioavailable copper, partly due to a lack of any other viable alternatives, such as

cation-exchange (Pagenkopf 1983, Playle and Dixon 1994a). Yet it has several disadvantages over a cation-exchange method. While modelling does begin to account for biological influences on metal speciation, and thus bioavailability, it is seriously constrained by its dependence on accurate and appropriate binding constants, as well as an accurate and thorough quantification of *all* the potentially interacting components present, including the ligands of sensitive resident organisms, dissolved and suspended solids, and DOC (Neubecker and Allen 1983). This in turn depends on a thorough understanding of the structure and function of all components, and with the gill, identification of a binding constant that is toxicologically relevant. Meeting *all* these constraints is time consuming, and it is often difficult and quite expensive to quantify all the chemical parameters necessary for geochemical modeling. So although they may provide adequate estimates of bioavailable metal in some circumstances, their widespread practical applicability is debatable.

In contrast, a cation exchange resin's success is not dependent on difficult and time-consuming analyses of water quality parameters, nor on a vast array of geochemical binding constants necessary to describe the many chemical species possible in an aquatic environment. If this method can be successfully developed, the amount of metal removed from a water sample, expressed as a fraction of total metal, would represent the bioavailable component. Additionally, our broader hypothesis is that many aquatic organisms will have similar membrane binding characteristics to rainbow trout, because the gills and external membranes of numerous aquatic organisms such as daphnids, algae, and bacteria, respectively, may also have certain surface metal-binding constituents in common. This implies that a common definition of bioavailable copper is possible and that this definition could encompass a wide diversity of aquatic organisms. A single cation-exchange resin may then have broad applicability toward assessment of bioavailable metal for many key indicator species.

After an examination of several *commercially* available cation exchange resins (Amberlite IR-120 and DP-1, Bio-Rad CMBio-Gel) we conclude that none adequately simulate gill copper binding (Figure 12). Methods commonly employed to decrease resin binding strength and modify selectivity, such as increasing eluent flow rates or adding divalent or trivalent counterions, failed to sufficiently lower the apparent binding strength. The overwhelmingly high binding capacity of these resins, as compared to the gill, is the most reasonable explanation for these high apparent affinities (Rhom and Haas 1991, Bio-Rad 1992). As mentioned earlier, most cation exchange resins we have encountered are designed to efficiently remove a cation of interest, rather than selectively remove a particular form of the cation (i.e., an "all or nothing" action). This results in an extremely high apparent binding affinity (log $K \sim 10$), despite (surprisingly), quite low (log $K \sim 4$-7) absolute copper binding affinities. Therefore, on the logarithmic binding constant scale, the copper binding affinity of the fish gill is vastly lower than the typical resin binding affinity of log ABA = 10.3. Thus, use of such a column to fractionate bioavailable copper can greatly overestimate the true bioavailable metal fraction present (see Fernandez 1994).

Therefore, our next step was to synthesize a low capacity resin that simulates the copper binding capacity *and* the copper binding affinity of the fish gill. While the synthesized resins do more closely approximate the fish gill copper binding affinity than commercially available resins, they are still inappropriate models. Yet there remain many avenues for continued research aimed at synthesis of a suitable gill model. Glass bead diameter, varied organo-silane deposition concentrations, new organo-silane ligands, addition of "spacer" molecules to enhance stearic accessibility, and use of alternative support phases such as controlled pore glass are all viable alternatives in resin synthesis (Turkova 1993). The results presented thus far on the development and use of custom synthesized resins to measure the bioavailable fraction of aqueous metals are still preliminary, but they suggest that this technique may offer an extremely powerful approach to create a unique and valuable, biologically-relevant cation-exchange resin.

(c) Written Publications in Technical Journals, etc.

Hamelink, J.L., P.F. Landrum, H.L. Bergman, and W.H. Benson. (eds.). 1994. Bioavailability: Physical, Chemical, and Biological Interactions. CRC Press, Boca Raton, Florida. 239 pp.

MacRae, R.K. 1994. Toxicologically Relevant Copper Binding Affinities for Gills of Rainbow Trout (*Oncorhynchus mykiss*) and Brook Trout (*Salvelinus fontinalis*). M.S. Thesis, University of Wyoming, Laramie, Wyoming. (In preparation for defense of thesis, December, 1994).

Smith, D.E. 1994. The Copper Binding Characteristics of Trout Mucus: Possible Consequences for Toxicity. M.S. Thesis, University of Wyoming, Laramie, Wyoming. (In preparation for defense of thesis, December, 1994).

MacRae, R.K., D.E. Smith, N.G. Swoboda-Colberg, J.S. Meyer, and H.L. Bergman. 1994. Toxicologically relevant copper binding affinities for gills of rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*). (In preparation for submission to Environmental Toxicology and Chemistry).

Smith, D.E., R.K. MacRae, C.J. Boese, J.S. Meyer, and H.L. Bergman. 1994. The copper binding characteristics of trout mucus: Possible consequences for toxicity. (In preparation for submission to Environmental Toxicology and Chemistry).

Boese, C.J., D.E. Smith, R.K. MacRae, and H.L. Bergman. 1994. Bioavailability of copper to *Daphnia magna*: An estimate based on binding affinity and capacity. (In preparation for submission to Environmental Toxicology and Chemistry).

(d) Professional Personnel Associated with Research

- Harold L. Bergman, Professor, Principal Investigator
- James I. Drever, Professor, Co-Principal Investigator
- Joseph D. Fernandez, Research Associate
- David D. Gulley, Research Associate
- Norbert G. Swoboda-Colberg, Research Associate
- Connie J. Boese, Research Associate
- Russell K. MacRae, Graduate Assistant (M.S., December, 1994)
- Darren E. Smith, Graduate Assistant (M.S., December, 1994)

(e) Interactions

(i) Papers Presented at Meetings, etc.

Bergman, H.L. 1992. Physiological mechanisms of metal toxicity to fish. American Fisheries Society, Western Section Annual Meetings, Fort Collins, Colorado. July, 1992. (Invited Presentation).

Bergman, H.L. 1992. Improving on empiricism: The potential value of a mechanistic understanding of bioavailability in regulating water quality. SETAC Pellston Aquatic Toxicology Workshop, Pellston, Michigan. August, 1992. (Invited Presentation).

MacRae, R.K., D.E. Smith, N.G. Swoboda-Colberg, and H.L. Bergman. 1992. Apparent copper binding affinity for rainbow trout gills. Society of Environmental Toxicology and Chemistry Annual Meetings, Cincinnati, Ohio. November, 1992. (Abstract in Proceedings).

Bergman, H.L. and C.M. Wood. 1993. Bioavailability and water quality criteria for heavy metals: A review of the problem and possible solutions. Society for Experimental Biology Annual Meetings, Canterbury, U.K. April, 1993. (Invited -- Abstract in Proceedings).

MacRae, R.K., D.E. Smith, N.G. Swoboda-Colberg, and H.L. Bergman. 1993. Apparent copper binding affinity for rainbow trout gills. Rocky Mountain Chapter of The Society of Environmental Toxicology and Chemistry Annual Meetings, Denver, Colorado. May, 1993. (Presentation).

Smith, D.E., and H.L. Bergman. 1993. The role of mucus in copper toxicity to rainbow trout. Rocky Mountain Chapter of The Society of Environmental Toxicology and Chemistry Annual Meetings, Denver, Colorado. May, 1993. (Presentation).

MacRae, R.K., D.E. Smith, N.G. Swoboda-Colberg, and H.L. Bergman. 1993. A competition bioassay determination of the apparent Cu binding affinity of trout gills. Society of Environmental Toxicology and Chemistry Annual Meetings, Houston, Texas. November, 1993. (Abstract in Proceedings).

Smith, D.E., R.K. MacRae, C.J. Boese, and H.L. Bergman. 1993. The copper binding characteristics of trout mucus: Possible consequences for toxicity. Society of Environmental Toxicology and Chemistry Annual Meetings. Houston, Texas. November, 1993. (Abstract in Proceedings).

(ii) Consultative and Advisory Functions to other Laboratories and Agencies including DoD

<u>Other Agencies, etc.</u>

- U.S. Environmental Protection Agency, Office of Water

  At the request of Margarete Heber and Chris Zarba, U.S. EPA Office of Water, Harold Bergman co-chaired an EPA sponsored workshop on "Aquatic Life Criteria for Metals" held in Annapolis, Maryland, from 25 to 29 January 1993. This workshop, which was attended by 40 invited participants and over 100 observers,

reviewed scientific and technical concerns about bioavailability of metals to aquatic biota, speciation of metals in water, and currently available analytical chemistry methods and geochemical speciation models for estimating the "bioavailable" fraction of metals. Results from the current AFOSR grant (91-0258) figured prominently in the workshop discussions. Several very important recommendations were presented to EPA from this workshop, including the following: (1) much of the currently available national monitoring data on metal concentrations in receiving waters is unreliable, and henceforth "clean" sampling and analytical methods must be employed to produce reliable data; (2) "dissolved" metal determinations approximate the "bioavailable" (thus, toxic) forms of most metals better than does the "total recoverable" determination; (3) scientific, technical and regulatory policy aspects of this metal "bioavailability" issue should be reviewed further by a group of experts in a workshop that specifically addresses copper, since this metal is currently causing one of the greatest regulatory problems in EPA's NPDES discharge permit program. EPA has already responded to the recommendation on dissolved metals by issuing an advisory to EPA Regional Offices and to state water quality programs on 1 October 1993 that "dissolved metal" could be used instead of "total recoverable metal" in determining compliance with water quality criteria and standards.

- Society of Environmental Toxicology and Chemistry (SETAC)

  Bergman co-chaired (with Margarete Heber from EPA) a special session at the 1993 Annual Meeting of SETAC in Houston entitled "Bioavailability of Metals and Aquatic Life Criteria". In this session, 10 invited speakers presented papers reviewing many of the issues discussed at the Annapolis workshop described above.

- Workshop on long-term research priorities to support aquatic life criteria for metals: Copper case study

  Bergman is the Chair of a Planning Committee to organize and host the copper workshop, recommended at the Annapolis Workshop as described above. Other members of the Committee are Dominic DiToro from Manhattan College, Herb Allen from the University of Delaware, Dave Hansen from EPA, and Jack Mattice from the Electric Power Research Institute. Currently, the workshop is being planned for the summer of 1995. The Planning Committee is now preparing a list of invited participants and soliciting funding from various government and industry groups concerned with water quality regulation for metals.

DoD Advisory Interactions

None

(f) New Discoveries, Inventions, etc.

None

(g) Other Information

Although this project has been plagued with personnel changes and delays, a number of significant accomplishments should be noted: (1) two M.S. students will have completed their degrees by December 1994, with funding from this project; (2) eight papers have been presented at regional, national and international meetings, with several of those papers contributing to an important on-going scientific and regulatory debate about how metals are regulated; (3) four or more refereed journal articles will be submitted for publication in December 1994, after completion of the two student theses; and (4) most important of all, even in advance of the publication of the results from this research, these results along with earlier work in this laboratory are influencing an important change in regulatory policy by the U.S. EPA, which may result in more defensible regulations based on "bioavailable metals" rather than the over-protective "total recoverable metals".

And although we were not completely successful in developing and validating a workable cation exchange chromatography procedure to match the binding affinity of copper to fish gills and other aquatic organism surfaces, we have had considerable success in working toward this goal. We have evaluated a number of commercial cation exchange resins and consulted extensively with the technical staff of commercial suppliers of cation exchange resins, and we have been able to rule out the commercial resins because they all have apparent binding affinities for copper that are three or more orders of magnitude higher than the copper binding affinities of aquatic biota. We have also evaluated the possibility of synthesizing custom cation exchange resins to match the copper binding affinities for aquatic biota, and we are now very close to meeting this objective. Our research efforts are continuing and we have every reason to expect success in developing and validating a cation exchange chromatography procedure that can be used to measure the "bioavailable" fraction of copper in natural surface waters. If we are as successful as we expect, we believe that this approach would also work for other regulated metals, including aluminum, cadmium, lead, silver, and zinc.

(h) References

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